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Award Number: DAMD17-00-1-0347

TITLE: Role of IRS-1 Phosphorylation in IGF-1 and IL-4 Signaling in Breast Cancer

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REPORT DATE: June 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual Summary (1 Jun 00-31 May 03)

4. TITLE AND SUBTITLE
Role of IRS-1 Phosphorylation in IGF-1 and IL-4 Signaling in Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0347

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Insulin-like growth factor-1 (IGF-I) stimulates proliferation of MCF-7 cells via the type I IGF receptor (IGF1R) and causes phosphorylation of the adaptor protein, insulin receptor substrate-1 (IRS-1). Interleukin 4 (IL-4) inhibits breast cancer cells and also phosphorylates IRS-1. The hypothesis was that IGF-I and IL-4 phosphorylate different residues with IGF-I treatment targeting IRS-1 for degradation via the proteasome. The goal of this project was to identify amino acids of IRS-1 phosphorylated by IGF-I compared to IL-4 using two-dimensional electrophoresis (2DE) of phosphorylated IRS-1. However, as reported in the two previous reports, several technical difficulties and limitations of 2DE were encountered and impeded the completion of the tasks. In the final year, work was continued on 2DE of IRS-1 but despite sustained efforts, it has not been possible to detect IRS-1 after 2DE. Unfortunately, it appears that the large size, abundance in cells, and perhaps charge of IRS-1 may make it difficult to use this technique. As the long-term objective of my training was to identify new targets for breast cancer therapy and inhibit IGF action, I have successfully worked on two other projects – one to inhibit IGF-I action in breast cancer cells using a humanized single chain antibody against IGF1R and a second project to inhibit metastasis of cancer cells using a truncated IGF1R that I have shown behaves in a dominant negative manner.

14. SUBJECT TERMS
Insulin-like growth factor-1 (IGF-I), interleukin-4 (IL-4), IRS-1, breast cancer cell growth inhibition

15. NUMBER OF PAGES
61

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

ANNUAL SUMMARY REPORT

INTRODUCTION:

Insulin like growth factor-1 (IGF-1) is a potent mitogen for breast cancer cells and the IGF system is a key growth regulatory pathway in breast cancer. Binding of IGF-1 to the type I IGF receptor (IGFIR) stimulates a cascade of signaling events leading to cell growth. IGF-1 transduces its signal through the adaptor protein insulin receptor substrate-1 (IRS-1). IGF-1 treatment results in phosphorylation of IRS-1. The cytokine interleukin-4 (IL-4) inhibits breast cancer cells. Our laboratory has shown that IL-4 acting via its receptor stimulates programmed cell death and also phosphorylates IRS-1. Thus, two factors with opposing effects on breast cancer cells cause phosphorylation of IRS-1. We have shown that IGF-1 causes a higher level of phosphorylation of IRS-1 than IL-4. In addition, IGF-1 causes acute phosphorylation of IRS-1 with rapid loss of detectable IRS-1 while IL-4 causes sustained phosphorylation of IRS-1. Phosphorylation of IRS-1 by IGF-1 causes degradation of IRS-1 by the ubiquitin proteasome pathway.

My post-doctoral project proposed to study the mechanisms by which IRS-1 is coupled with growth stimulatory pathways with the aim of answering the question: How can phosphorylation of IRS-1 be involved in both cell growth and cell death? The hypothesis was that IRS-1 is differentially phosphorylated by IGF-1 and IL-4 in breast cancer cells and that the phosphorylation of IRS-1 by IGF-1 results in phosphorylation of specific serine/threonine residues which targets IRS-1 for degradation via the ubiquitin pathway. This hypothesis was to be tested by identifying the tyrosine, serine and threonine residues of IRS-1 phosphorylated by IGF-1 and IL-4. This was to be done using two-dimensional gel electrophoretic separation of phosphorylated IRS-1 followed by phosphoaminoacid analysis and sequencing of phosphopeptides that differ between IGF-1 and IL-4 stimulation of IRS-1. Thus, the goal of this project was to identify those residues of IRS-1, which are phosphorylated by IGF-1 and not IL-4 and are, thus, responsible for targeting IRS-1 for degradation and then make a mutant IRS-1 that can neutralize the action of IGF-1 or may be change a mitogenic signal to a death signal.

BODY:

As I had mentioned in my annual summary reports of June 2001 and June 2002, several technical difficulties were encountered during two-dimensional Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel electrophoresis (2DE) of IRS-1 and the tasks listed in the Statement of Work have not been accomplished. It is becoming increasingly evident that due to the limitations of 2DE (1), it may not be possible to successfully use 2DE for large proteins such as IRS-1, which is a 185 kDa protein.

As stated in the two previous annual summary reports, 2DE of IRS-1 was performed using:

-First dimension isoelectric focusing (IEF) with 11 cm immobilized IPG strips of pH 3-10 (Amersham Biosciences, Piscataway, NJ).

-Second dimension SDS-PAGE using 8% Tris-Glycine gels.

Despite several attempts I was unable to detect IRS-1 by immunoblotting after 2DE.

In the last year of this training grant, time was spent on trying yet other conditions to optimize 2DE for large proteins. Various combinations of solubilization and reducing agents such as thiourea, tributyl phosphine, and dithioerythritol were tried in order to reduce the protein and maximize its solubility for 2DE. In addition, I continued to test several antibodies against IRS-1 and several new anti-phosphotyrosine antibodies but none detected IRS-1 or phosphorylated IRS-1 after 2DE. Unfortunately, our results suggest that the large size, abundance in cells, and perhaps charge of IRS-1 may make it difficult to use this technique.

Thus, in addition to the experiments outlined in the fellowship training grant I have also worked on two other projects with the goal of inhibiting IGF action in breast cancer cells. In years 2 and 3, I have used a humanized single chain antibody against the type I IGF1R to inhibit breast cancer cells. I have shown that this antibody makes breast cancer cells refractory to the mitogenic effects of IGF-I and that it does so by downregulation of the receptor. These results have shown that IGF1R is indeed a suitable target for breast cancer therapy. A manuscript describing the mechanism of action of this antibody (2) was published in the February 2003 issue of *Cancer Research* (see list of Reportable Outcomes). I am currently studying the effects of this antibody *in vivo* using an MCF-7 xenograft model of breast cancer. I believe that with additional preclinical data, we may be able to test the efficacy of this antibody in a clinical study. In the past year, I have also worked on a third project investigating the role of IGF1R in breast cancer cell proliferation, motility and metastasis. I have used a C-terminally truncated IGF1R which behaves in a dominant negative manner. This truncated receptor was stably transfected into a metastatic variant of MDA-MB-435 cells, LCC6 cells (3) and the effect of the truncated dominant negative IGF1R on breast cancer proliferation and metastasis has been investigated. This manuscript has been submitted to *Journal of Biological Chemistry* (4).

The tasks for year 3 of this grant in the original Statement of Work were:

Task 9: Generate stable clones of MCF-7 cells carrying selected mutant IRS-1 and empty vector (Year 2, months 11-12, Year 3, months 1-2).

This task could not be accomplished as residues on IRS-1 that were differentially phosphorylated by IGF-I compared to IL-4 could not be identified due to the technical limitations of 2DE as described. Therefore, stable clones of MCF-7 cells carrying mutant IRS-1 were not generated. However, as part of my project on the role of IGF1R in cancer metastasis and the effect of IGF1R inhibition on metastasis, stable clones of LCC6 cells expressing a truncated IGF1R were generated. I was able to show that this truncated IGF1R behaved in a dominant negative manner to inhibit metastasis. A manuscript describing this work has been submitted to the *Journal of Biological Chemistry* (4) in May 2003.

Task 10: Assess *in vitro* growth, both anchorage dependent and anchorage independent, in response to IGF-I and IL-4 treatment (Year 3, months 3-10).

This task was not accomplished using cells expressing mutant IRS-1. However, as described under Task 9 above, I worked on another project to determine the effect of a truncated IGF1R on growth and metastasis of cells expressing a C-terminally truncated IGF1R. I have performed experiments to determine the effect of this truncated IGF1R on anchorage-dependent and anchorage-independent growth *in vitro*, motility, *in vivo* growth in athymic mice, and metastasis of cell lines expressing truncated IGF1R as part on the study on the effect of IGF1R on cancer metastasis

Task 11: Analyze and prepare data for publication and presentation at national meeting, and prepare manuscripts for publication (Years 2 and 3, months 11-12)

I consider that this task has been accomplished fully as I have presented data at several meetings this year (see Reportable Outcomes). I have also published a manuscript on the mechanism of action of a single chain antibody. A manuscript on the role of the IGF1R in cancer metastasis was submitted to *Journal of Biological Chemistry* in May 2003 and a revised version has been resubmitted (4). Thus, even though the experiments in the original Statement of Work were not accomplished, I have published my work on two other projects. In fact, tasks 9 and 10 listed here were successfully done on the third project that I have worked on – the role of IGF1R in metastasis.

Furthermore, as an alternate approach to determine the residues or motifs that may target IRS-1 for degradation, two hemagluttinin A (HA) tagged mutants of human IRS-1 have been obtained from Dr. Adrian Lee (Baylor College of Medicine, Houston, TX). These mutants delete varying regions of IRS-1 from the C-terminal end. The last couple months of this year were spent in generating stable cells lines expressing these deletion mutants of IRS-1. In the next few months, clones will be screened and will be tested for their effect on the degradation of IRS-1 by the proteasome and growth of breast cancer cells. During year 3 of this training grant, I chose to spend more time on my other two projects in order to generate publications which is important for my career as an independent investigator.

KEY RESEARCH ACCOMPLISHMENTS:

- *In vitro* and *in vivo* effects of a single chain antibody against IGF1R on IGF mediated growth
- Role of IGF1R in metastasis using a dominant negative IGF1R and the demonstration that inhibition of IGFIR in breast cancer cells inhibits their metastasis

REPORTABLE OUTCOMES:

1. Attended the Era of Hope Department of Defense Breast Cancer Research Program Meeting in September 2002 and gave a Symposium Platform Presentation titled "A Humanized Single Chain Antibody Against the Type I IGF Receptor Renders Breast Cancer Cells Refractory to the Mitogenic Effects of IGF-I." on September 26, 2002 in the session "New Approaches to Tyrosine Kinase Growth Factor Receptors".

2. Gave an oral presentation titled "Downregulation of the type I insulin-like growth factor receptor by a chimeric single chain antibody *in vitro* and *in vivo*" at the 9th Annual San Antonio Breast Cancer Symposium on December 11, 2002.

3. Selected for an Astra-Zeneca Scholar Award at the San Antonio Breast Cancer Symposium in December 2002.

4. Abstract submitted for the Gordon Conference on "IGFs in physiology and disease" was selected for an oral presentation in March 2003 at Ventura, CA.

   A dominant negative type I insulin-like growth factor-1 receptor (IGF-IR) inhibits metastasis of breast cancer cells.
   Deepali Sachdev, Julie S. Hartell, Adrian V. Lee[1], Xihong Zhang, and Douglas Yee. Department of Medicine and Cancer Center, University of Minnesota, MN 55455 and [1]Breast Center, Baylor School of Medicine, Houston, TX.

5. Attended a Scientific Advisory Board meeting of Novartis Pharmaceuticals as a consultant for their 'IGF1R Inhibitor Program' and presented my work on inhibition of IGF-I action in breast cancer at the Advisory Board meeting in Short Hills on April 26, 2003.

6. Published a manuscript in *Cancer Research* on my work on inhibition of IGF action in breast cancer cells using a single chain antibody against IGF1R (see attached appendix).

   Sachdev, D., Li, S., Hartell, J.S., Fujita-Yamaguchi, Y., Miller, J.S., and Yee, D. 2003. A chimeric humanized single-chain antibody against the Type I insulin-like growth factor receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I. Cancer Research 63: 627-635.

7. Co-author on a review article in *Clinical Cancer Research.*

   Lee, A.V., Schiff, R., Cui, X., **Sachdev, D.**, Yee, D., Gilmore, A.P., Streuli, C.H., Oesterreich, S., Hadsell, D.L. 2003. New mechanisms of signal transduction inhibitor action: receptor tyrosine kinase down-regulation and blockade of signal transactivation. Clinical Cancer Research 9 :516S-23S.

8. Manuscript submitted to *Journal of Biological Chemistry* is under revision for publication (see attached appendix).

   Sachdev, D., Hartell, J.S., Lee, A.V., Zhang, X., and Yee, D. A dominant negative type I insulin-like growth factor-1 receptor inhibits metastasis of human cancer cells. (in revision for *JBC*)

9. Submitted a manuscript to *Molecular Cancer Therapeutics* in collaboration with another lab.

   Ye, J-J., Liang, S-J., Guo, N., Li, S-L., **Sachdev, D.,** Yee, D., Brunner, N., Ikle, D., and Fujita-Yamaguchi, Y. Combined effects of anti-IGF-I receptor single chain antibody, scFv-Fc, and tamoxifen on breast tumor growth *in vivo.*

CONCLUSIONS:

While the original proposed statement of work could not be completed due to the technical limitations of 2DE, I have successfully worked on two other projects that also allowed me to get training in breast cancer research. The overall goal of my post-doctoral training was to inhibit IGF-I action in breast cancer and prepare me for a career in breast cancer research. The long-term goal of my project was to identify suitable targets for treatment of breast cancer. Towards that end, I feel that I have made some novel contributions to the role of IGF in breast cancer and the identification of new targets for therapy.

Even though the tasks in the original proposal were not accomplished, I feel that this post-doctoral training gave me the opportunity to study the inhibition of IGF-I action in breast cancer and to obtain training for an independent career. In the coming year I hope to use the data generated from the work supported by this grant to apply for other funding with the aim of establishing myself as an independent investigator.

I would like to thank the Department of Defense's Breast Cancer Research Program for supporting my training by awarding me this Post-doctoral training grant.

REFERENCES:

1. Jenkins, R. E. and Pennington, S. R. 2001. Arrays for protein expression profiling: towards a viable alternative to two-dimensional gel electrophoresis? Proteomics 1: 13-29.

2. Sachdev, D., Li, S., Hartell, J. S., Fujita-Yamaguchi, Y., Miller, J. S., and Yee, D. 2003. A chimeric humanized single-chain antibody against the Type I insulin-like growth factor receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I. Cancer Research 63: 627-635.

3. Leonessa, F., Green, D., Licht, T., Wright, A., Wingate-Legette, K., Lippman, J., Gottesman, M. M., and Clarke, R. 1996. MDA435/LCC6 and MDA435/LCC6MDR1: ascites models of human breast cancer, Br J Cancer. *73:* 154-61.

4. Sachdev, D., Hartell, J. S., Lee, A. V., Zhang, X., and Yee, D. A dominant negative type I insulin-like growth factor-1 receptor inhibits metastasis of human cancer cells. (in revision for *JBC*)

APPENDIX:

1: Reprint of manuscript published in *Cancer Research.*
2. Copy of manuscript submitted to *JBC.*

[CANCER RESEARCH 63, 627–635, February 1, 2003]

A Chimeric Humanized Single-Chain Antibody against the Type I Insulin-like Growth Factor (IGF) Receptor Renders Breast Cancer Cells Refractory to the Mitogenic Effects of IGF-I[1]

Deepali Sachdev, Shu-Lian Li, Julie S. Hartell, Yoko Fujita-Yamaguchi, Jeffrey S. Miller, and Douglas Yee[2]

Department of Medicine, University of Minnesota Cancer Center, Minneapolis, Minnesota 55455 [D. S., J. S. H., J. S. M., D. Y.]; Molecular Biology Division, Beckman Research Institute of the City of Hope, Duarte, California 91010 [S-L. L.]; and Department of Applied Biochemistry, School of Engineering, Tokai University, Hiratsuka 259-1292, Japan [Y. F-Y.]

ABSTRACT

Insulin-like growth factors (IGFs) stimulate breast cancer proliferation, motility, and survival. The type I IGF receptor (IGF1R) mediates the effects of IGF-I. Thus, inhibition of IGF1R activation could inhibit IGF action in breast cancer cells. A single-chain antibody directed against IGF1R (IGF1R scFv-Fc) has been shown to partially inhibit xenograft growth of MCF-7 cells in athymic mice. In this study, we have examined the effects of scFv-Fc on IGF1R signaling in the estrogen receptor-positive (ER+) MCF-7 breast cancer cells *in vitro* and *in vivo*. The antibody stimulated IGF1R activation *in vitro* in MCF-7 cells and was unable to block IGF-I effects. The antibody also stimulated proliferation of MCF-7 cells in monolayer growth assays. To determine how scFv-Fc could stimulate *in vitro* growth yet inhibit *in vivo* tumor growth, we examined the effect of scFv-Fc on IGF1R expression. In MCF-7 cells, scFv-Fc down-regulated IGF1R levels after 2 h, and the levels were greatly reduced after 24 h. In contrast, IGF-I treatment over the same time period did not affect IGF1R levels. Twenty-four-h pretreatment of cells with scFv-Fc blocked IGF-I mediated phosphorylation of insulin receptor substrate-1 and subsequent extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 and phosphatidylinositol 3′-kinase activation. In contrast, cells treated with 5 nM IGF-I for 24 h still retained the ability to further activate downstream signaling pathways in response to IGF-I. Moreover, pretreatment of MCF-7 cells with scFv-Fc rendered them refractory to further proliferation induced by additional IGF-I. Twenty-four-h pretreatment of cells with scFv-Fc also inhibited IGF-I stimulated anchorage-independent growth. scFv-Fc did not enhance antibody-dependent cell-mediated cytotoxicity. *In vivo*, treatment of mice bearing MCF-7 xenograft tumors with scFv-Fc resulted in near complete down-regulation of IGF1R. Our data show that scFv-Fc stimulates biochemical activation of IGF1R, then causes receptor down-regulation, making MCF-7 cells refractory to additional IGF-I exposure. These results indicate that such chimeric single-chain antibodies against IGF1R have future potential in breast cancer therapy by causing down-regulation of receptor.

INTRODUCTION

Some breast cancer cells proliferate in response to the IGFs.[3] In other cells, IGFs enhance motility and survival (1, 2). These effects are mediated by the IGF1R, which consists of two covalently linked polypeptide chains, each with an extracellular α-subunit and a transmembrane β-subunit that contains tyrosine kinase activity. The IGF1R is transported to the membrane fully assembled in the dimeric form and after ligand binding, tyrosine kinase activity is initiated. Activation of this receptor protects breast cancer cells from chemotherapy (3, 4), causes radioresistance (5), and may be required for oncogenic transformation and tumorigenicity of cells (6). Since the kinase activity of IGF1R has been reported to be enhanced in breast cancer (7), it may be a potential target for therapy (8). The success of trastuzumab, a humanized antibody against HER2/c-erbB2 (9, 10), in the treatment of breast cancer patients (11) and the small molecule tyrosine kinase inhibitor imatinib mesylate (STI-571) that blocks the kinase activity of the Bcr-Abl oncoprotein and c-kit tyrosine kinase in the treatment of chronic myelogenous leukemia (12, 13) and gastrointestinal stromal tumors (14) have proved that antigrowth factor receptor and tyrosine kinase inhibition therapy have important clinical benefits.

IGF1R has been reported to be important in several different cancers, including breast cancer, prostate cancer, liver cancer, glioblastomas, and childhood malignancies, and several approaches have been used to inhibit signaling via IGF1R. An antisense IGF1R oligodeoxynucleotide-based therapy is currently in clinical trial in patients with astrocytomas (15). A second way to inhibit IGF1R is the use of small molecule inhibitors that would inhibit the tyrosine kinase activity by binding to the ATP binding site or substrate binding site in the kinase domain of IGF1R or by blocking substrate binding to the activated receptor. Several small molecule inhibitors of IGF1R are currently being studied, but to date, there are no reported inhibitors specific for IGF1R (16, 17). Unlike other growth factor receptor tyrosine kinases, IGF1R is only activated by ligands; overexpression alone does not lead to its activation as is seen in the case of Her2/neu (18, 19). Therefore, any approaches that prevent binding of ligands to IGF1R may inhibit activation of the receptor.

Several antibodies have been studied as a strategy to inhibit IGF1R activation. Among these, the monoclonal antibody αIR3 (20), which blocks binding of IGF-I to IGF1R, has been widely studied. αIR3 inhibits proliferation of MCF-7 cells *in vitro* (21, 22). It also inhibits the growth of some breast cancer cell lines such as MDA-MB-231 and T61 *in vivo* (23) but not the xenograft growth of IGF responsive cells such as MCF-7 (24).

In this study, we have used a single-chain antibody that inhibits the binding of IGF-I and IGF-II to IGF1R (25) to determine its effect on IGF1R signaling. The anti-IGF1R scFv-Fc (referred to as scFv-Fc) used in this study was engineered from a mouse monoclonal antibody, 1H7, that blocks binding of IGF-I and IGF-II to IGF1R (26). scFv-Fc is a chimeric antibody that has the Fc domain of human IgG1 fused to the Fv region of the mouse monoclonal antibody 1H7. scFv-Fc has been shown to partially inhibit the xenograft growth of MCF-7 cells (25).

In MCF-7 cells, we have shown that IGF-I activates IGF1R, which phosphorylates the adaptor protein IRS-1 (27). IRS-1 then recruits other signaling molecules, resulting in the activation of further downstream pathways including the MAPK and PI3K pathways. The ob-

Received 5/20/02; accepted 11/21/02.

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[1] This work was supported by USAMRMC Grant DAMD17-00-1-0347 (to D. S.), NIH Grant R01 CA74285 and PHS Cancer Center Support Grant P30 CA77398 (to D. Y.), and California Breast Cancer Research Program Grant 3CB-0186 and a grant from Tokai University (to Y. F-Y.).

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[3] The abbreviations used are: IGF, insulin-like growth factor; IGF1R, type I IGF receptor; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3′-kinase; HRP, horseradish peroxidase; ERK, extracellular signal-regulated kinase; IGF1Rβ, β-subunit of IGF1R; IGFBP-1, IGF-binding protein 1; IMEM, Improved Minimum Essential Medium; SFM, serum-free media; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ADCC, antibody-dependent cell-mediated cytotoxicity; NK, natural killer.

jective of this study was to identify the mechanism of action of scFv-Fc on IGF1R function in MCF-7 cells. We found that scFv-Fc acted as a full agonist in MCF-7 cells and activated IGF1R tyrosine kinase activity to stimulate monolayer growth. However, scFv-Fc, but not IGF-I, down-regulated levels of IGF1R over time. Cells exposed to scFv-Fc became refractory to additional IGF stimulation, supporting a role for this antibody as an anti-IGF therapeutic.

MATERIALS AND METHODS

Reagents. All reagents and chemicals were purchased from Sigma (St. Louis, MO), and cell culture reagents were from Invitrogen/Life Technologies, Inc. (Rockville, MD) unless otherwise noted. IGF-I was purchased from Gro*Pep* (Adelaide, Australia), and the anti-IGF1R scFv-Fc was engineered and purified as described previously (25). Antiphosphotyrosine antibody (PY-20) conjugated to HRP was from BD Transduction Laboratories (Lexington, KY). Antibodies against ERK1/ERK2 (phosphospecific and total) and Akt (phosphospecific and total) were purchased from Cell Signaling (Beverly, MA). The rabbit polyclonal antibody against IRS-1 was produced by Alpha Diagnostics (San Antonio, TX) by immunizing rabbits with a 14-amino acid peptide (YASINFQKPEDRQ) from the COOH-terminal region of IRS-1. The immune serum was affinity purified on immobilized protein A column using the ImmunoPure IgG purification kit (Pierce, Rockford, IL). The polyclonal antibody against IGF1Rβ was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antirabbit secondary antibody conjugated to HRP was from Amersham Biosciences (Piscataway, NJ). Protein A-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acrylamide, bis-acrylamide, and prestained molecular weight markers were from Bio-Rad (Hercules, CA). Recombinant human IGFBP-1 expressed in *Escherichia coli* (28) was originally obtained from Synergen (Boulder, CO).

Cell Lines and Culture. MCF-7 cells were obtained from Dr. C. Kent Osborne (Baylor College of Medicine, Houston, TX) and were routinely maintained in IMEM with Zinc Option (Richter's modification) with 5% fetal bovine serum, 11.25 nM human insulin (Eli Lilly, Indianapolis, IN), 50 units/ml penicillin, and 50 μg/ml streptomycin. NIH3T3 cells overexpressing IGF1R, NIH3T3/IGF1R (29), were obtained from Dr. Reiner Lammers (Department of Internal Medicine IV, Tübingen, Germany) and grown in DMEM supplemented with 10% fetal bovine serum.

Cell Stimulation. MCF-7 cells were grown in 10-cm dishes in regular growth media. Confluent cells (70%) were washed twice with PBS and serum deprived for 24 h in SFM as described previously (27). For treatment with IGF-I or scFv-Fc, medium was replaced with SFM containing 5 nM IGF-I or 250 nM scFv-Fc for times as indicated in the figure legends. To determine whether scFv-Fc inhibited IGF-I mediated activation of IGF1R or other pathways, cells were first pretreated with scFv-Fc for 30 min or 24 h and then with IGF-I for an additional 10 min. To determine whether down-regulation of IGF1R by scFv-Fc occurs by the proteasome pathway, cells were pretreated with 30 μM MG115 (Calbiochem, San Diego, CA), a proteasome inhibitor, for 2 h before treatment with IGF-I or scFv-Fc. To determine the effect of a lysomotropic agent on scFv-Fc mediated down-regulation of IGF1R, cells were pretreated with 40 mM methylamine for 4 h before treatment with IGF-I or scFv-Fc for various times.

Cell Lysis. Cells were washed three times with ice-cold PBS on ice and lysed with 500 μl/10-cm dish lysis buffer TNESV [50 mM Tris-Cl (pH 7.4); 1% NP40; 2 mM EDTA (pH 8.0); 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin]. Lysates were clarified by centrifugation at 12,000 $\times$ *g* for 20 min at 4°C, and soluble cellular proteins were used for experiments or stored at −20°C. Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL).

Immunoblotting. For immunoblotting, 40 μg of cellular proteins were subjected to reducing SDS-PAGE on 8% polyacrylamide gels after the Laemmli system (30). After SDS-PAGE, proteins were transferred to nitrocellulose. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 [TBST − 0.15 M NaCl; 0.01 M Tris-HCl (pH 7.4); 0.05% Tween 20] for 1 h at room temperature with gentle shaking. Membranes were then washed five times with TBST for 5 min each. For detecting phosphorylated proteins, membranes were incubated with 1:10,000 dilution of PY-20 antiphosphotyrosine antibody in TBST for 1 h at room temperature. Membranes were washed five times with TBST for 5 min each. Chemiluminescence was then performed as described below. Membranes were also blotted with either 1:2000 dilution of a rabbit polyclonal antibody against IRS-1affinity purified on immobilized protein A or 1:1000 dilution of rabbit polyclonal antibody against IGF1Rβ (Santa Cruz Biotechnology, Inc.). Phospho-Akt (Ser^{473}), Akt, phospho-p44/p42 ERK1/ERK2 (Thr^{202}/Tyr^{204}) and total ERK1/ERK2 antibodies were used as per manufacturer's instructions. Membranes were washed as before and incubated with 1:2000 dilution of antirabbit secondary antibody conjugated to HRP for 1 h at room temperature. Membranes were washed as before and chemiluminescence was done using Super-Signal West Pico substrate (Pierce, Rockford, IL).

Immunoprecipitations. A total of 500 μg of total cellular proteins was first precleared with 25 μl of protein A-agarose for 30 min and then incubated overnight with 3 μl of polyclonal serum against IRS-1. A total of 25 μl of protein A-agarose was added for 4 h. All steps were done on a rocker at 4°C. Immune complexes were collected by centrifugation at 12,000 $\times$ *g* for 1 min. Immunoprecipitates were washed five times with 500-μl each TNESV by resuspension and centrifugation. After the final wash, immunoprecipitates were resuspended in 30 μl of TNESV and 30-μl 2$\times$ Laemmli sample buffer containing 30 mg/ml DTT. Samples were boiled for 5 min, centrifuged, and supernatants were subjected to SDS-PAGE.

Proliferation Assays. Cells were plated in 24-well plates with 20,000 cells/well in regular growth medium. Cells were switched to SFM for 24 h and then treated as indicated in the figure legends. All treatments were done in triplicates. Growth was measured 4–5 days after treatment. Growth was assayed by the MTT assay as described previously (31). A total of 60 μl of 5 mg/ml MTT solution in PBS was added to each well. After incubation for 4 h at 37°C, wells were aspirated and formazan crystals were lysed with 500 μl of solubilization solution (95% DMSO +5% IMEM). Absorbance was measured with a plate reader at 570 nm using a 670-nm differential filter.

Anchorage-independent Growth. Anchorage-independent growth assays were performed as described previously (28). A total of 1×10^4 cells/well of a 6-well plate was used. One ml of 0.8% SeaPlaque agarose (BioWhitaker, Rockland, ME) in culture medium was solidified in the bottom of each well as the bottom agar. Cells in growth media without or with 5 nM IGF-I, 250 nM scFv-Fc, or both IGF-I and scFv-Fc were mixed with 0.45% agarose. The cells mixed with agarose were overlaid on the bottom agar. After 12–14 days, colonies were counted using a light microscope with a grid in the eyepiece. The grid has 100 divisions and colonies larger than two-thirds of each square in the grid were counted. Three randomly selected fields were counted for each well, and the average number of colonies is shown. Results shown are representative of one experiment with triplicates for each treatment.

ADCC Assay. MCF-7 and SKBR-3 cells were labeled with ^{51}Cr by incubating the cell pellets with 40 μCi of ^{51}Cr for 1 h at 37°C. Human NK cells isolated from normal volunteer were used as effectors in the assay. Peripheral blood mononuclear cells were obtained from normal human blood by density gradient centrifugation using Ficoll-Paque. NK cells were isolated from peripheral blood mononuclear cell using a MACS NK cell isolation kit (Miltenyi Biotec, Auburn, CA) by depletion of non-NK cells according to the manufacturer's instructions. The purity of the NK cells was determined by flow cytometric analysis using CD56-PE and CD3-FITC and was >85% $CD56^+/CD3^-$ for all experiments. A total of 50,000 NK cells was mixed with 5000 ^{51}Cr-labeled cells to give an effector:target ratio of 10:1 and incubated with various concentrations of either trastuzumab or scFv-Fc for 4 h. Each antibody dilution was tested in triplicate. The ability of the antibodies to cause cell lysis was measured by counting the released ^{51}Cr. The results are shown as percentage of lysis plotted against the concentration of the antibodies.

Animal Studies and Xenograft Tumor Extract Analyses. Sixty-day release pellets of 17β-estradiol (Innovative Research of America, Sarasota, FL) were implanted s.c. into 4-week-old female athymic mice. Each mouse was implanted with a 0.5-mg pellet. The next day, 5×10^6 MCF-7 cells in serum-free IMEM (without phenol-red) were injected into mammary fat pads on each side of each mouse. Tumor growth was measured weekly. When MCF-7 xenograft tumors were established (3 weeks), tumors from the left side of all mice were resected before treatment. Five mice were then injected i.p. with 500 μg of scFv-Fc in PBS, and 5 mice were injected with PBS as control. Twenty-four h after injection of PBS or scFv-Fc, mice were sacrificed, and the remaining tumors were harvested. The tumors were snap frozen in liquid

nitrogen. Frozen tumor samples were homogenized in a tissue pulverizer in a dry ice/ethanol bath. Tissue homogenates were suspended in 500-μl lysis buffer TNESV. Homogenates were centrifuged at 12000 × g for 20 min, and supernatants were stored at −70°C. One hundred μg of each tumor extract along with 40 μg of MCF-7 cell lysate were subjected to reducing SDS-PAGE on 8% acrylamide gels followed by immunoblotting for IGF1Rβ.

RESULTS

scFv-Fc Does Not Inhibit IGF-I Induced Phosphorylation of IRS-1 in MCF-7 Cells. Since the scFv-Fc has been shown to block IGF-I binding to IGF1R and partially inhibit xenograft growth of MCF-7 cells in mice, we first examined the effect of scFv-Fc on activation of IRS-1 in MCF-7 cells. In MCF-7 cells, we have previously shown that IRS-1 is the major adaptor protein phosphorylated by IGF-I-mediated activation of IGF1R. As reported previously (27) and shown in Fig. 1*A*, IGF-I treatment resulted in detection of a 185-kDa phosphorylated band in IGF-I-treated cells (Fig. 1*A*, *Lane 2*) but not in untreated cells (Fig. 1*A*, *Lane 1*). Pretreatment of cells with 250 nM scFv-Fc before stimulation with IGF-I did not inhibit IGF-I-induced phosphorylation of IRS-1 (*Lane 3* compared with *Lane 2*) in Fig. 1*A*. Surprisingly, treatment with 250 nM scFv-Fc alone (Fig. 1*A*, *Lane 4*) resulted in phosphorylation of 185-kDa IRS-1 protein in a manner similar to IGF-I. As a control, cells were also treated with IGFBP-1. We have previously shown that IGFBP-1 blocks IGF-I action (28, 32) and as shown in Fig. 1*A*, *Lane 5*, pretreatment of cells with 8-fold molar excess of IGFBP-1 blocked IGF-I-mediated phosphorylation of IRS-1. IGFBP-1 treatment alone does not result in phosphorylation of IRS-1 (Fig, 1*A*, *Lane 6*). The lower panel shows that the total levels of IRS-1 were unchanged by treatment with scFv-Fc.

As both IRS-1 and IRS-2 have the same relative molecular mass, we wanted to confirm that the 185-kDa phosphorylated protein in cells treated with scFv-Fc was IRS-1. Therefore, cell lysates were immunoprecipitated with IRS-1 antibody followed by antiphosphotyrosine blotting (Fig. 1*B*). As shown in Fig. 1*B*, *Lane 4*, scFv-Fc treatment indeed resulted in phosphorylation of IRS-1 in MCF-7 cells similar to IGF-I (Fig. 1*B*, *Lane 2*). As a control in Fig. 1*B*, *Lane 5*, IGFBP-1 inhibited IGF-I-mediated phosphorylation of IRS-1 in MCF-7 cells.

scFv-Fc and IGF-I Activate Similar Downstream Signaling Pathways. We next determined whether scFv-Fc inhibited signaling pathways distal to IRS-1. We and others have shown that activation of

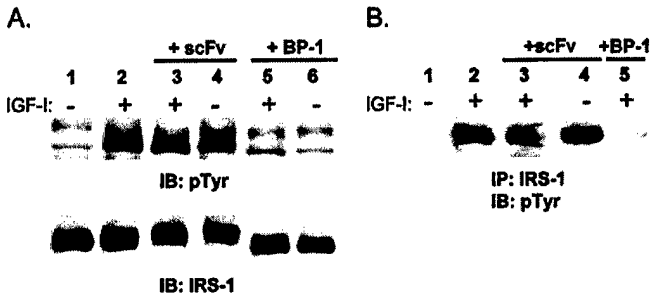

Fig. 1. scFv-Fc activates IRS-1 phosphorylation and does not block IGF-I effects. Seventy percent confluent MCF-7 cells were switched to SFM as described in "Materials and Methods." Twenty-four h later, cells were treated with 5 nM IGF-I or 250 nM scFv-Fc for 10 min. *A*, *top panel*, anti-phosphotyrosine immunoblot; *bottom panel*, IRS-1 immunoblot. Cells were either untreated (*Lane 1*), treated with 5 nM IGF-I (*Lane 2*), pretreated with scFv-Fc for 30 min prior to 5 nM IGF-I (*Lane 3*), or 250 nM scFv-Fc (*Lane 4*). As controls, in *Lane 5* cells were pretreated with 40 nM IGFBP-1 for 30 min before stimulation with IGF-I, and in *Lane 6*, cells were treated with IGFBP-1 alone. *B*, immunoprecipitation with IRS-1 followed by immunoblotting with antiphosphotyrosine antibody. *Lane 1* shows untreated cells, and *Lane 2* shows cells treated with IGF-I. *Lane 4* is cells treated with scFv-Fc alone.

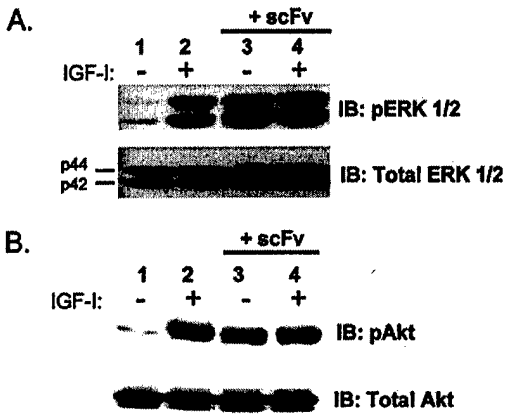

Fig. 2. scFv-Fc and IGF-I activate similar signaling pathways. *A*, activation of ERK1/ERK2 of the MAPK family. Cells were treated as described for Fig. 1 and then immunoblotted for activated ERK1/ERK2 (*top panel*) and total ERK1/ERK2 (*bottom panel*). Both the p42 and p44 kDa ERK1/ERK2 are shown. *B*, activation of Akt/PKB. Cellular proteins were immunoblotted for phosphorylated Akt (*top panel*) and total Akt (*bottom panel*). In both *A* and *B*, cells were untreated (*Lane 1*), treated with 5 nM IGF-I (*Lane 2*), or 250 nM scFv-Fc for 10 min (*Lane 3*). In *Lane 4*, cells were pretreated with scFv-Fc for 30 min before being stimulated with IGF-I for 10 min.

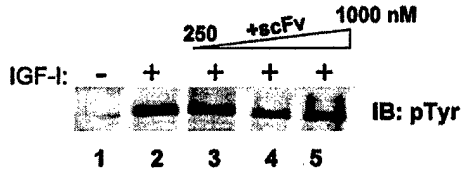

Fig. 3. scFv-Fc does not inhibit IGF-I-mediated effects, even at increasing concentrations. MCF-7 cells were pretreated with various concentrations of scFv-Fc for 30 min before stimulation with 5 nM IGF-I for 10 min. Cellular proteins were then immunoblotted for antiphosphotyrosine. Cells were untreated (*Lane 1*) or treated with 5 nM IGF-I for 10 min (*Lane 2*). Cells were also pretreated with 250 nM (*Lane 3*), 500 nM (*Lane 4*), or 1000 nM (*Lane 5*) scFv-Fc for 30 min before IGF-I treatment.

IGF1R by IGF-I results in phosphorylation of p44/p42 (ERK1/ERK2) of the MAPK pathway (27) and activation of the PI3K pathway (27, 33). We were interested in determining whether scFv-Fc perhaps inhibited IGF-I-induced activation of these downstream signaling pathways, which are linked to mitogenesis in MCF-7 cells. Thus, MCF-7 cells were treated with scFv-Fc before stimulation with or without IGF-I, and activation of ERK1/ERK2 was measured using a phosphospecific p44/p42 antibody. scFv-Fc did not inhibit IGF-I-induced phosphorylation of p44/p42 ERK1/ERK2 (Fig. 2*A*, *Lane 4 versus Lane 2*). Furthermore, scFv-Fc alone induced phosphorylation of ERK1/ERK2 (Fig. 2*A*, *Lane 3*). The time course of ERK1/ERK2 activation by scFv-Fc was similar to that by IGF-I (data not shown).

We then examined the PI3K pathway in MCF-7 cells. Measurement of phosphorylated Akt, a substrate for PI3K, was used to measure activation of this pathway. scFv-Fc did not inhibit IGF-I-mediated phosphorylation of Akt as shown in Fig. 2*B* (*Lane 4*). Similar to the case with ERK1/ERK2, scFv-Fc also resulted in phosphorylation of Akt as shown in *Lane 3* of Fig. 2*B*. However, scFv-Fc caused a sustained phosphorylation of Akt compared with IGF-I (data not shown). Thus, biochemically, scFv-Fc activated IGF1R and downstream signaling pathways in MCF-7 cells *in vitro* in a manner similar to IGF-I.

To rule out the possibility that the inability of scFv-Fc to inhibit IGF-I induced phosphorylation of IRS-1 and activation of downstream signaling pathways was because of suboptimal concentrations of scFv-Fc, MCF-7 cells were pretreated with increasing concentrations of scFv-Fc from 250 to 1000 nM before stimulation with 5 nM

IGF-I. scFv-Fc at either 500 nM (as shown in Fig. 3, *Lane 4*) or 1000 nM (Fig. 3, *Lane 5*) did not inhibit IGF-I induced phosphorylation of IRS-1. This indicated that the inability of scFv-Fc to block IGF-I was not a concentration-dependent effect because even a 200-fold molar excess of scFv-Fc did not inhibit activation of IRS-1 in MCF-7 cells.

scFv-Fc Stimulates Activation of IGF1R and Downstream Signaling Molecules in Cells Overexpressing IGF1R. It has been reported that the inhibitory or stimulatory behavior of some antibodies may be dependent on cell surface receptor number (9, 34). To determine whether perhaps the stimulatory behavior scFv-Fc was dependent on the density of IGF1R in the cells, we used a NIH3T3 cell line that stably overexpresses IGF1R (NIH3T3/IGF1R). NIH3T3/IGF1R cells express ~1000-fold more receptors/cell compared with MCF-7 cells (25). As with MCF-7 cells, the ability of scFv-Fc to inhibit IGF-I mediated activation of IGF1R in these cells was studied by treating cells with scFv-Fc for 30 min before stimulation with IGF-I. In addition, NIH3T3/IGF1R cells were also treated with 250 nM scFv-Fc for 10 min to examine its effect on IGF1R in these cells. As these cells overexpress IGF1R, both phosphorylated IRS-1 (185 kDa) and phosphorylated IGF1R (97 kDa β-subunit) were seen on an antiphosphotyrosine immunoblot after IGF-I treatment (Fig. 4*A*, *Lane 2*). In NIH3T3/IGF1R cells, scFv-Fc did not inhibit IGF-I mediated activation of IGF1R as shown in Fig. 4*A*, *Lane 3* or further downstream ERK1/ERK2 (Fig. 4*C*, *Lane 3*) or PI3K pathway (Fig. 4*D*, *Lane 3*). scFv-Fc alone also activated IGF1R (Fig. 4*A*, *Lane 4*), ERK1/ERK2 of the MAPK pathway (Fig. 4*C*, *Lane 4*), and Akt (Fig. 4*D*, *Lane 4*). Fig. 4*B* shows that the total levels of IRS-1 are unchanged by treatment with scFv-Fc (*Lane 4*).

scFv-Fc Causes Degradation of IRS-1. IGF-I causes ubiquitination followed by proteasomal mediated degradation of IRS-I after 2 h, and levels of IRS-1 are greatly reduced by 8 h of IGF-I treatment (35). We assayed the effect of scFv-Fc treatment on levels of IRS-1. As shown in Fig. 5, scFv-Fc resulted in down-regulation of IRS-1 when examined by immunoblot, consistent with the effects of IGF-I.

scFv-Fc Stimulates *in Vitro* Proliferation of MCF-7 Cells. Because scFv-Fc behaved as a biochemical agonist of IGF1R in MCF-7 cells, we next examined its effect on *in vitro* proliferation of MCF-7 cells. Not surprisingly, 250 nM scFv-Fc also stimulated the proliferation of MCF-7 cells to a similar extent as 5 nM IGF-I as shown in Fig. 6. This assay measures the ability of cells to proliferate for a short-term in serum-free conditions in response to growth factors or mitogens while attached to tissue culture plates. Although this assay measures a different phenotype than xenograft growth in mice, it nevertheless shows a discrepancy in the effect of scFv-Fc on IGF-I mediated mitogenic response of MCF-7 cells *in vitro* compared with *in vivo* growth in athymic mice.

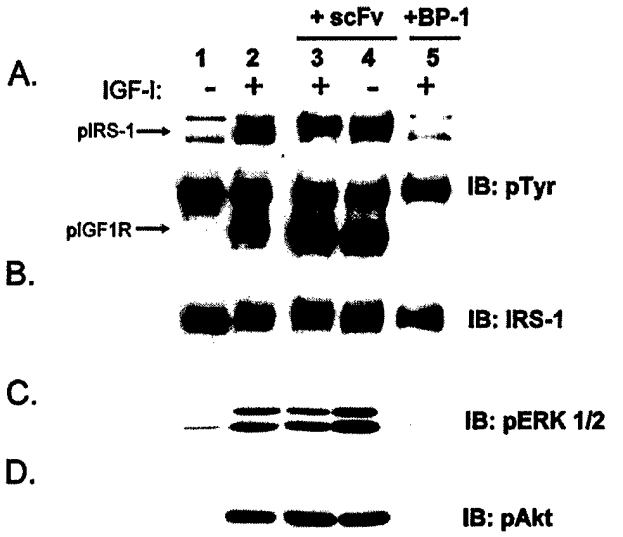

Fig. 4. Activation of IGF1R by scFv-Fc is not dependent on cell receptor number. NIH3T3 cells overexpressing IGF1R were treated as described in Fig. 1, and cell lysates were immunoblotted for phosphotyrosine (*A*), IRS-1 (*B*), phosphoERK1/ERK2 (*C*), and phosphoAkt (*D*). Cells were either untreated (*Lane 1*), treated with 5 nM IGF-I (*Lane 2*), pretreated with scFv-Fc for 30 min before 5 nM IGF-I (*Lane 3*), or treated with 250 nM scFv-Fc (*Lane 4*). In *Lane 5*, as a control to show that signaling pathways activated by IGF-I can be blocked, cells were pretreated with IGFBP-1 for 30 min before stimulation with IGF-I.

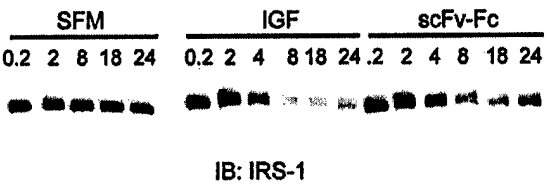

Fig. 5. scFv-Fc causes degradation of IRS-1. MCF-7 cells were left untreated (*lanes labeled SFM*) or treated with 5 nM IGF-I (*lanes labeled IGF*) or 250-nm scFv-Fc (*lanes labeled scFv-Fc*) for times as indicated in the figure. Cell lysates were then immunoblotted for total levels of IRS-1.

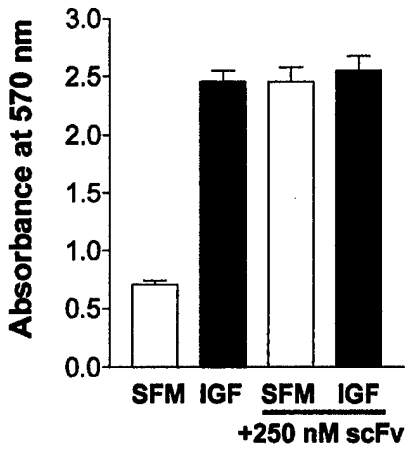

Fig. 6. scFv-Fc stimulates *in vitro* proliferation of MCF-7 cells. *In vitro* proliferation assay was performed using the MTT assay. MCF-7 cells in serum-free conditions were treated with IGF-I, scFv-Fc or IGF and scFv-Fc together for 5 days. Proliferation was then measured by the uptake of the MTT reagent followed by measurement of absorbance at 570 nm. Growth is shown as the absorbance at 570 nm and the data represent the mean ± SE of four independent experiments with triplicate samples in each experiment.

scFv-Fc Stimulates Anchorage-independent Growth of MCF-7 Cells. To further explore the growth effects of scFv-Fc, we examined the ability of the antibody to inhibit IGF-I stimulated anchorage-independent growth. Perhaps the scFv-Fc could have a partial inhibitory effect on the xenograft growth of MCF-7 cells by affecting anchorage-independent growth. The results of the anchorage-independent growth assay in Fig. 7 are shown as the average number of colonies from triplicate wells. scFv-Fc did not inhibit IGF-I stimulated anchorage-independent growth of MCF-7 cells, but the antibody stimulated anchorage-independent growth, similar to the results in monolayer growth assays.

scFv-Fc Does Not Enhance ADCC. As scFv-Fc is a chimeric antibody with the Fc region of human IgG1 fused to the scFv, the results obtained *in vivo* could be because of activation of ADCC. Indeed, this mechanism of xenograft growth inhibition has been shown in trastuzumab-treated animals (36). NK cells have receptors for Fc, and this could be one of the mechanisms by which scFv-Fc partially inhibited tumor growth (37). Athymic mice, although deficient in T cells, have elevated levels of NK cells (38). We, therefore, performed an *in vitro* ^{51}Cr release cytotoxicity assay using MCF-7

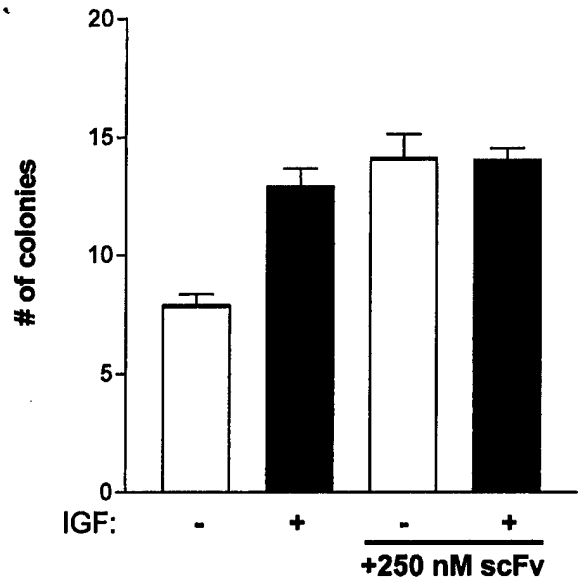

Fig. 7. scFv-Fc stimulates anchorage-independent growth. MCF-7 cells without or with 5 nM IGF-I or 250 nM scFv-Fc were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates. Using a grid, colonies formed were counted on a portion of the well. Three randomly selected fields were counted for each well and averaged. Each treatment was done in triplicate, and the results are shown as the average number of colonies ± SE. The experiment was repeated three times with similar results, and a representative experiment is shown.

cells as targets and NK cells purified from human peripheral blood mononuclear lymphocytes. As a positive control, we used trastuzumab along with SKBR3 cells, which overexpress HER2/c-erbB2. As shown in Fig. 8, scFv-Fc did not enhance lysis of MCF-7 cells or SKBR3 cells. The positive control trastuzumab caused increased lysis of both MCF-7 and SKBR3 targets.

scFv-Fc Causes Down-Regulation of IGF1R. In an attempt to determine the mechanism by which scFv-Fc could partially inhibit xenograft growth of MCF-7 in mice yet stimulate *in vitro* proliferation, we measured the levels of IGF1R after treatment of MCF-7 cells with scFv-Fc compared with IGF-I. Cells were treated with 5 nM IGF-I, 250 nM scFv-Fc, or both for different time periods. IGF1R levels were measured by immunoblotting with an antibody against the β-subunit of IGF1R. Untreated cells in *Lane 1* (for each time point) showed no change in IGF1R levels from 10 min to 24 h as seen in Fig. 9*A*. Similarly, IGF-I treatment from 10 min to 24 h did not alter levels of IGF1R (Fig. 9*A*, *Lane 2* compared with *Lane 1*). In contrast, scFv-Fc caused a rapid down-regulation of IGF1R after 2 h as shown in Fig. 9*A*, *Lane 3*, and by 24 h, very little IGF1R was detected. This effect of scFv-Fc in down-regulating IGF1R appeared to be a dominant effect as cells treated with IGF-I and scFv-Fc together (Fig. 9*A*, *Lanes 4*) also showed a marked down-regulation of IGF1R.

As many signaling molecules are regulated by ubiquitination followed by degradation by the proteasome, we investigated whether the mechanism by which scFv-Fc down-regulates IGF1R is via the proteasome pathway. We used the proteasome inhibitor MG115 (39) to block the proteolytic activity of the proteasomes. Cells were pretreated with 30 μM MG115 for 2 h and then with IGF-I or scFv-Fc for 20 h. As shown in Fig. 9*B*, MG115 did not inhibit down-regulation of IGF1R by scFv-Fc (*Lane 3*; MG115 treatment) compared with cells treated with only scFv-Fc (*Lane 3*, no pretreatment). This suggests that down-regulation of IGF1R by scFv-Fc does not occur via the proteasomal pathway.

To determine whether down-regulation of IGF1R was mediated by the endocytic pathway, cells were pretreated with 40 mM methylamine (40) and then treated with IGF-I or scFv-Fc for various times. Methylamine raises pH of endosomes and inhibits the endocytic pathway. Pretreatment of cells with methylamine for 4 h blocked scFv-Fc-mediated down-regulation of IGF1R as seen in lanes marked 3 (for each time point) in Fig. 9*C* compared with cells that were not pretreated with methylamine (*Lanes 3* in *A*). This suggests that down-regulation of IGF1R by the scFv-Fc occurs via the lysosomal/endocytic pathway as increasing the endosomal pH using the lysomotropic agent methylamine inhibited receptor down-regulation.

Pretreatment of Cells with scFv-Fc Causes Inhibition of IGF-I Mediated Phosphorylation of IRS-1 and Activation of MAPK and PI3K Pathways. If the down-regulation of IGF1R by scFv-Fc was responsible for the *in vivo* growth inhibition of MCF-7 cells, we hypothesized that cells should not respond to additional IGF-I stimulation after antibody induced down-regulation of IGF1R. To determine whether this was the case, we pretreated cells with either IGF-I or scFv-Fc for 24 h. After this pretreatment, cells were left either untreated (lanes labeled SFM) or stimulated with additional 5 nM IGF-I. In Fig. 10*A*, *Lanes 1–3* show that both IGF-I and scFv-Fc treatment resulted in phosphorylation of IRS-1 compared with untreated cells (*Lane 1*). When cells were pretreated with IGF-I for 24 h and then treated with additional 5 nM IGF-I for 10 min, they still

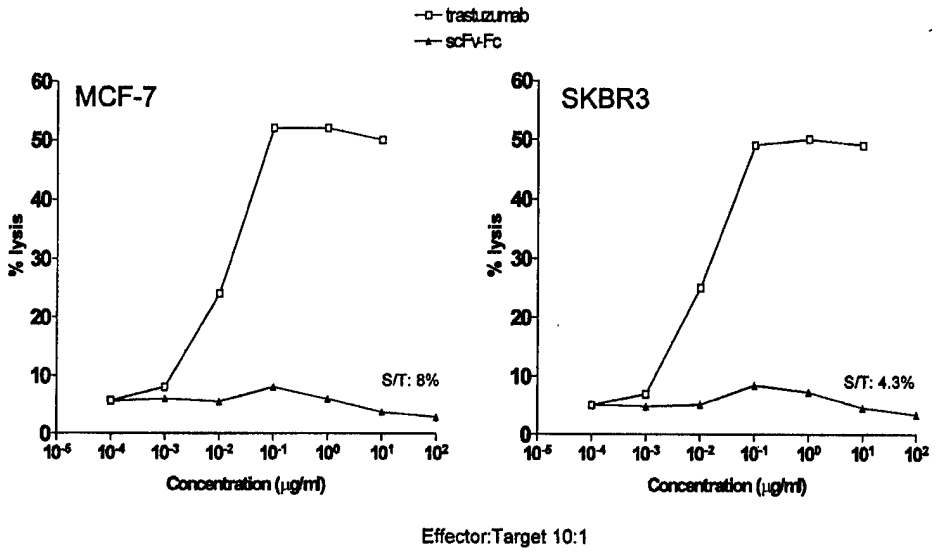

Fig. 8. scFv-Fc does not enhance ADCC. ^{51}Cr-labeled MCF-7 or SKBR3 cells were mixed with human NK cells at a ratio of 1:10 and incubated with various concentrations of scFv-Fc (▲) or trastuzumab (□) for 4 h. Cells were harvested, and cpm of ^{51}Cr released by lysis was counted using a γ-counter. Percentage of lysis of cells was calculated using Sp-S/T-S, where Sp is the specific lysis caused by antibody, S is the spontaneous lysis of cells in the absence of antibody, and T is the total lysis of cells by Triton X-100. Spontaneous lysis of cells was <8%. The results are plotted as percentage of lysis against the concentration of antibody. The data shown is representative of three experiments with similar results.

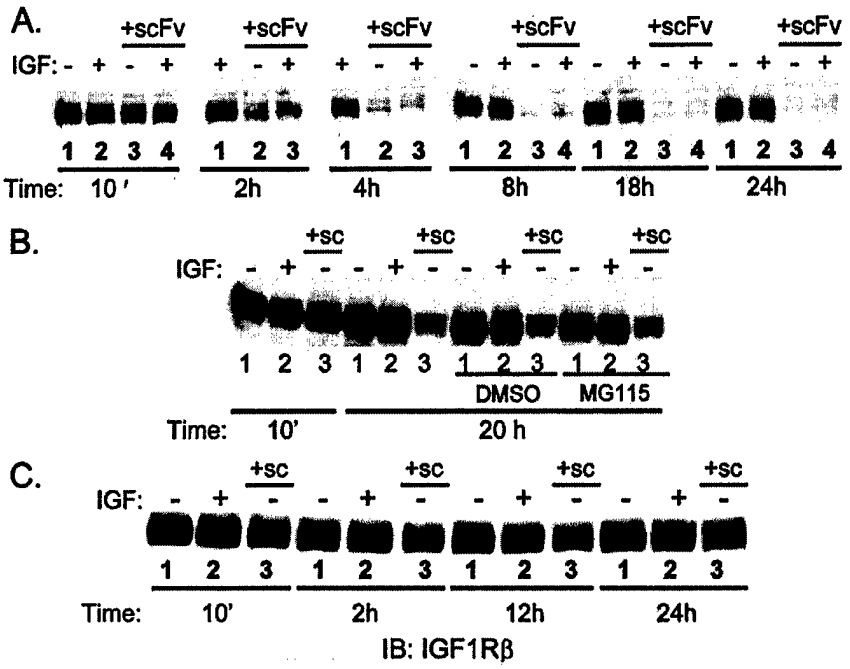

Fig. 9. scFv-Fc causes rapid down-regulation of IGF1R via the endocytic pathway. MCF-7 cells were left untreated (*lanes labeled 1*) or treated with 5 nM IGF-I (*Lanes 2*), 250 nm scFv-Fc (*Lanes 3*), or IGF-I + scFv-Fc (*Lanes 4*) for various times as indicated below the lane number. In *B*, cells were pretreated with 30 μM MG115 in DMSO or DMSO alone for 2 h and then treated with IGF-I or scFv-Fc for 20 h. In *C*, cells were pretreated with 40 mM methylamine for 4 h before treatment with IGF-I or scFv-Fc for various times. Cell lysates were then immunoblotted for total levels of IGF1Rβ. The M_r 97,000 β-subunit of IGF1R is shown in the figure.

retained the ability to phosphorylate IRS-1 (Fig. 10*A*, *Lane 5* compared with *Lane 4*). In contrast, when cells were pretreated with scFv-Fc for 24 h followed by treatment with 5 nM IGF-I for 10 min, phosphorylated IRS-1 was not detected (Fig. 10*A*, *Lane 7*). Cells pretreated with scFv-Fc then re-treated with additional antibody did not have enhanced IRS-1 phosphorylation (data not shown). Furthermore, as shown in Fig. 10, pretreatment of cells with scFv-Fc for 24 h before treatment with IGF-I for 10 min also blocked subsequent phosphorylation of p44/p42 ERK1/ERK2 (Fig. 10*B*, *Lane 7*) and Akt (Fig. 10*C*, *Lane 7* compared with *Lane 6*). In contrast, pretreatment with IGF-I for 24 h does not inhibit stimulation of these pathways. Additional IGF-I still leads to activation of both the ERK1/ERK2 (Fig. 10*B*, *Lane 5*) and PI3K pathways (Fig. 10*C*, *Lane 5*).

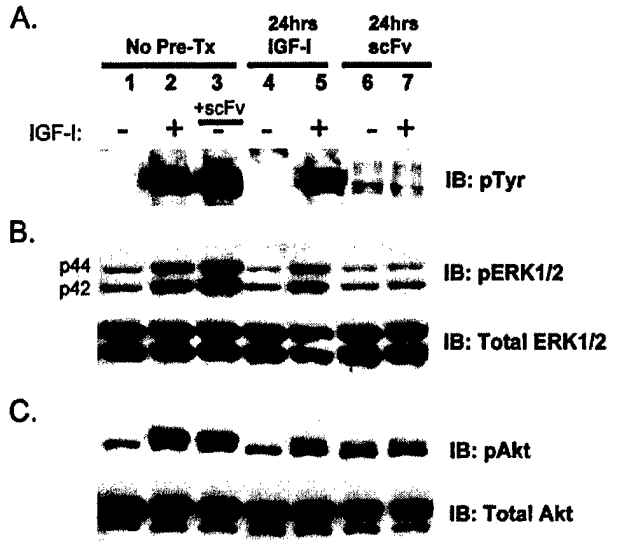

Fig. 10. Pretreatment of cells with scFv-Fc inhibits phosphorylation of IRS-1 and activation of downstream signaling pathways induced by IGF-I. *A*, antiphosphotyrosine immunoblot. *B*, phospho-ERK1/ERK2 immunoblot. *C*, phospho-Akt immunoblot. Cells were pretreated with either IGF-I (*lanes labeled 24 h IGF*) or scFv-Fc (*lanes labeled 24 h scFv*) for 24 h. After this pretreatment, cells were either left untreated (*lanes labeled −IGF-1*) or stimulated with 5 nM IGF-I (*lanes labeled +IGF-1*). *Lanes 1–3* show that both IGF-I and scFv-Fc phosphorylate IRS-1 compared with untreated cells (*Lane 1*). *Lanes 4* and *5* show cells pretreated with IGF-I for 24 h and then treated with an additional 5 nM IGF-I for 10 min (*Lane 5*) or untreated (*Lane 4*). *Lanes 6* and *7* show cells pretreated with scFv-Fc for 24 h and then treated with 5 nM IGF-I for 10 min (*Lane 7*) or untreated (*Lane 6*). When cells are pretreated with scFv-Fc for 24 h followed by treatment with 5 nM IGF-I for 10 min, they no longer phosphorylate IRS-1 (*Lane 7* compared with *Lane 2*). Furthermore, pretreatment of cells with scFv-Fc for 24 h also blocked subsequent phosphorylation of p44/p42 ERK1/ERK2 (*B*, *Lane 7*) and phosphorylation of Akt (*C*, *Lane 7* compared with *Lane 6*) by IGF-I.

Thus, 24-h pretreatment of cells with scFv-Fc inhibited the ability of 5 nM IGF-I to phosphorylate IRS-1 and blocked subsequent ERK1/ERK2 and PI3K activation. In contrast, cells treated with 5 nM IGF-I for 24 h still retained the ability to be additionally stimulated by subsequent treatment with additional IGF-I. These data show that down-regulation of IGF1R by scFv-Fc blocked the ability of IGF-I to initiate mitogenic signaling.

Pretreatment of Cells with scFv-Fc Makes Them Refractory to Additional IGF-I Mediated Proliferation. Since scFv-Fc caused down-regulation of IGF1R and inhibition of IGF-I-mediated activation of ERK1/ERK2 and PI3K pathways, we tested if 24-h pretreatment of cells with scFv-Fc before exposure to additional IGF-I would have any effect on the growth of cells. As shown in Fig. 5, scFv-Fc stimulated MCF-7 cells to proliferate. Here, we were interested in studying the further response of cells to additional IGF-I after 24-h pretreatment with scFv-Fc. Cells were pretreated with IGF-I or scFv-Fc for 24 h. After this pretreatment, cells were washed and incubated with or without additional IGF-I. Cell growth was measured after 4 days. Pretreatment with IGF-I and treatment with subsequent IGF-I resulted in increased proliferation compared with the control as shown in Fig. 11. In contrast, cells pretreated with sc-Fv-Fc did not proliferate further in response to additional IGF-I.

IGF1R down-regulation by scFv-Fc also inhibited anchorage-independent growth of MCF-7 cells. As shown in Fig. 12, cells pretreated with IGF-I for 24 h had increased colony formation in soft agar in response to IGF-I. In contrast, cells pretreated with scFv-Fc had decreased colony formation.

Thus, the difference between these results and that in Figs. 6 and 7 relates to whether or not IGF1R was present on the cell surface when the cells were exposed to additional IGF-I. Although scFv-Fc activates receptor initially, when cells are re-treated with IGF-I, the

scFv-Fc treated cells do not respond, whereas the IGF-I treated cells still have ample receptor on the cell surface allowing for additional increase in proliferation.

scFv-Fc Causes Down-Regulation of IGF1R *in Vivo* in Xenograft Tumors in Mice Injected with the Antibody. To confirm if our *in vitro* findings of down-regulation of IGF1R levels by scFv-Fc was responsible for inhibition of tumor growth *in vivo*, we tested the effect of scFv-Fc on IGF1R levels in mice with MCF-7 xenograft tumors. Ten mice bearing two xenograft tumors each, one on either side, were studied. Tumors were resected from the left side of all 10 mice before treatment. The next day, 5 mice received i.p. injections of 500 μg of scFv-Fc, and 5 mice received i.p. injections of control PBS. To allow sufficient time for scFv-Fc to cross the peritoneum, 24 h after treatment, mice were sacrificed and tumors harvested. All tumor extracts were then subjected to immunoblotting for IGF1R. Fig. 13 shows that all MCF-7 xenograft tumors had high levels of IGF1R before treatment (lanes labeled pre-treatment in the top and bottom panel). Twenty-four h after treatment, tumors taken from animals treated with scFv-Fc had significant down-regulation of IGF1R (bottom panel, lanes labeled 24 h post-treatment). No change in IGF1R was observed in PBS-treated animals (top panel). Densitometric analysis showed that after treatment with scFv-Fc, IGF1R levels in xenograft tumor extracts were ~22% (n = 5) of that in tumor extracts before scFv-Fc treatment. In contrast, in mice that received control PBS injections, the IGF1R levels were ~99% of that before treatment.

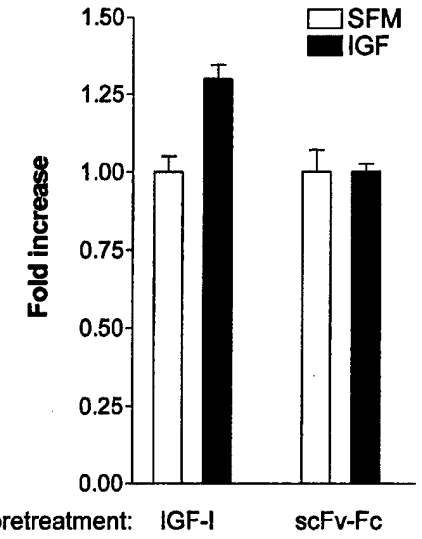

Fig. 11. Pretreatment of cells with scFv-Fc renders them refractory to additional growth stimulation by IGF-I. *In vitro* proliferation assay was performed using the MTT assay. MCF-7 cells in serum-free conditions were pretreated with either IGF-I (bars labeled 24 h pretreatment with IGF) or scFv-Fc (bars labeled 24 h pretreatment with scFv) for 24 h. Media were removed, and cells were washed and then treated without (SFM) or with 5 nM IGF-I (IGF-I) for 5 days. Proliferation was then measured by the uptake of the MTT reagent followed by measurement of absorbance at 570 nm. Growth is shown as the fold increase over control and the data represent the mean ± SE of triplicate readings from two experiments.

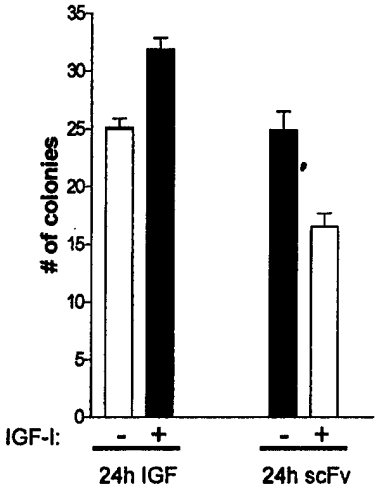

Fig. 12. Pretreatment of cells with scFv-Fc inhibits IGF-I-stimulated colony formation in soft agar. Cells were pretreated with scFv-Fc or IGF-I for 24 h. MCF-7 cells were then washed, trypsinized, centrifuged, and resuspended in medium. Cells without or with 5 nM IGF-I were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates. Colonies formed were counted after 12 days using a microscope with a grid. Three randomly selected fields were counted for each well and averaged. Each treatment was done in triplicate, and the results are shown as the average number of colonies ± SE. Similar results were obtained when the experiment was repeated twice, and a representative experiment is shown.

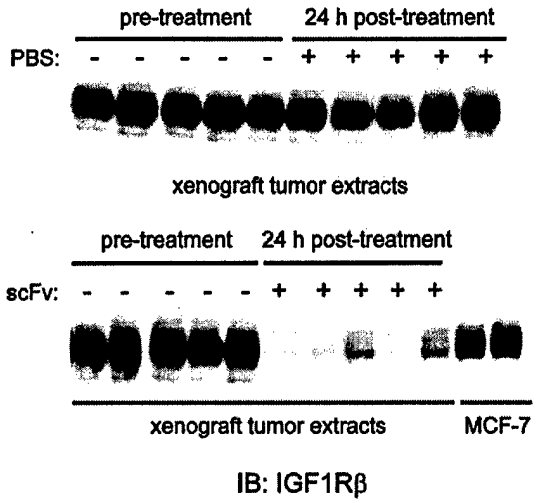

Fig. 13. scFv-Fc down-regulates IGF1R *in vivo*. Female athymic mice bearing MCF-7 xenograft tumors on each side were studied. Tumors from the left side were resected from each mouse before treatment. The next day, 5 mice received i.p. injections of 500 μg of scFv-Fc, and 5 mice received i.p. injections of PBS. Twenty-four h after treatment, tumors were collected. Tumor samples were frozen in liquid nitrogen and homogenized in TNESV buffer using a pulverizer as described in "Materials and Methods." A total of 100 μg of tumor extracts and 40 μg of MCF-7 cell lysate (*bottom panel; lanes labeled MCF-7*) were immunoblotted for total IGF1Rβ levels. Total IGF1R levels in the tumor extracts in the 10 mice before treatment is shown in lanes labeled pre-treatment in the *top* and *bottom panels*. IGF1R levels in the mice that were injected with scFv-Fc is shown in lanes labeled 24 h post-treatment in the *bottom panel*. Tumor extracts from mice after treatment with PBS is shown in the *top panel*.

DISCUSSION

The IGF system had been implicated in various roles in carcinogenesis. A truncated dominant negative IGF1R has been reported to inhibit the metastasis of MDA-MB-435 breast cancer cells (41, 42). In breast cancer patients, tumors with high IGF1R levels have increased ipsilateral breast recurrence of tumor after lumpectomy and radiation (5). Antisense IGF1R strategies are being tested in astrocytomas and neuroblastomas (43).

Thus, IGF1R is being tested as a target for cancer therapeutics. An obvious strategy to disrupt IGF-I action would be to develop small molecule inhibitors of the tyrosine kinase activity of IGF1R. Whereas small molecule inhibitors such as Iressa (ZD1839) have been successfully used to inhibit the tyrosine kinase activity of EGFR (44, 45) and also HER2, it has been difficult to do the same with IGF1R. IGF1R shares a high degree of homology to the insulin receptor (46), making it difficult to design small molecule tyrosine kinase inhibitors specific for IGF1R. Although some such inhibitors have been described, they all have the ability to inhibit the insulin receptor (17). The determi-

nation of higher resolution crystal structures of the tyrosine kinase domain of IGF1R (47) may enhance the design of a tyrosine kinase inhibitor that is highly specific for IGF1R.

However, antibodies that can be recombinantly engineered to specifically target growth factor receptors are also a very attractive option. The success of trastuzumab, a humanized monoclonal antibody against the extracellular domain of HER2/c-erbB2 receptor, in the treatment of breast cancer patients whose tumors overexpress HER2 supports this approach. A chimeric antibody developed against EGFR is currently in clinical trial (48). Here, we have examined a chimeric antibody against IGF1R that was engineered from an inhibitory monoclonal antibody, 1H7, that blocks binding of IGF-I and IGF-II to IGF1R (26). scFv-Fc was engineered by PCR and is a chimeric antibody that has the Fc domain of human IgG1 fused to the Fv region of the mouse monoclonal antibody 1H7.

We show here that scFv-Fc behaves as a biochemical agonist of IGF1R. It activates IGF1R and downstream signaling pathways in a fashion identical to IGF-I. There are other antibodies against IGF1R that also enhance receptor autophosphorylation (49). In the case of monoclonal antibodies against HER2/erbB2, several have been described that phosphorylate HER2 yet inhibit growth (50). Thus, there is no clear correlation between biochemical activation of receptor by an antibody and growth inhibitory properties (51). Surprisingly, scFv-Fc also stimulated *in vitro* growth of MCF-7 cells in short-term cultures. This was contrary to our expectation as we undertook these studies to understand the mechanism by which scFv-Fc inhibits *in vivo* growth of MCF-7 cells. Our *in vitro* monolayer and anchorage-independent growth experiments suggest that only a brief exposure of a stimulating ligand will result in apparent proliferation in these short-term assays. It is possible that activation of IGF1R signaling pathways, even for only a few hours, results in increased cell number when measured 5–7 days later. Thus, *in vitro* growth assays may not always be a good prediction of effects *in vivo*. Although the exact mechanism by which antibodies inhibit tumor growth is not understood, there is evidence that the *in vitro* and *in vivo* effects of antibodies can be opposing as has been reported for antibodies against ErbB2 (52). This may be because the *in vivo* effects of an antibody are also dependent on host-tumor interactions, and the tumor microenvironment also has an impact.

As scFv-Fc is a chimeric antibody and is partially humanized with the Fc region of human IgG1, it is possible that one of the mechanisms by which scFv-Fc inhibits xenograft growth of MCF-7 cells *in vivo* is by enhancing ADCC. However, our *in vitro* data suggest that ADCC is not the major mechanism by which scFv-Fc caused inhibition of tumor growth. Unlike trastuzumab, scFv-Fc did not enhance lysis of MCF-7 cells in an *in vitro* ADCC assay.

A second mechanism by which scFv-Fc could inhibit tumor growth is by its effect on IGF1R levels. scFv-Fc caused down-regulation of IGF1R both *in vitro* and in athymic mice with xenograft tumors that received 500 μg of scFv-Fc. The mechanism by which this occurs in not yet clear. The down-regulation of IGF1R does not occur via the proteasome-ubiquitin pathway as the proteasome inhibitor MG115 had no effect on scFv-Fc induced receptor down-regulation. In contrast, the lysomotropic agent methylamine blocked scFv-Fc induced down-regulation of IGF1R implicating the endosomal endocytic pathway. IGF-I treatment did not cause changes in steady state levels of IGF1R, although IGF-I also causes endocytosis of its receptor (53, 54). It is possible that IGF-I induced receptor endocytosis is balanced by receptor recycling to the membrane (55). In contrast, perhaps scFv-Fc causes increased endocytosis but may not allow for receptor recycling, causing a net decrease in cell surface receptor levels over time. Such an increase in receptor turnover leading to down-modulation of the oncogene product of HER2/c-erbB2 has been reported with a monoclonal antibody against the receptor (56). It does not appear that the tyrosine kinase activity of the receptor is required for IGF1R down-regulation because scFv-Fc also caused down-regulation of IGF1R in a metastatic variant of MDA-MB-435 cells, LCC6 cells (57), stably transfected with a truncated dominant negative IGF1R (data not shown). The antibody αIR3, which does not cause IGF1R tyrosine phosphorylation, also caused IGF1R down-regulation (data not shown). Although tyrosine kinase activation of the receptor is required for internalization of receptor by many monoclonal antibodies, it appears that tyrosine kinase activity is not always required for antibody-mediated down-regulation of IGF1R. It has been reported that tyrosine kinase activity is not required for internalization of a murine monoclonal antibody TA1 against HER2 (58).

It is possible that serine or threonine phosphorylation of IGF1R by scFv-Fc could contribute to IGF1R down-regulation. Xu *et al.* (50) have reported that a monoclonal antibody against HER2 that induces receptor down-regulation causes tyrosine phosphorylation of the receptor during short-term treatment and then causes serine phosphorylation of some intracellular substrates. It may also be that the scFv-Fc simply causes receptor aggregation leading to down-regulation.

Thus, *in vivo* repeated injection of scFv-Fc may cause sustained down-regulation of IGF1R, which results in inhibition of MCF-7 xenograft growth in mice. Using another mouse model of breast cancer, the T61 xenograft tumor model in athymic mice (59, 60), we have shown that i.p. injections of scFv-Fc resulted in decreased IGF1R levels in these tumors.[4]

A third mechanism may be that scFv-Fc alters the distribution of cell cycle components. It has been reported that cells that respond to IGF-I by a mitogenic response activate certain key cell cycle components (61). scFv-Fc may cause a decrease in the percentage of cells in the S phase, perhaps by induction of CDK2 kinase inhibitor $p27^{KIP1}$.

Given the fact that IGFs have pleiotropic effects on breast cancer, inhibition of IGF1R activation can be beneficial to breast cancer patients in several ways. Proliferation, cell metastasis, protection from chemotherapy, and radiation are all mediated by IGF1R. With recent reports indicating that activation of the IGF1R may be one of the mechanisms by which tumors become resistant to trastuzumab (62, 63), it may be even more important to target IGF1R as a potential breast cancer therapy. Our data show that down-regulation of IGF1R by a chimeric antibody could be a successful therapeutic strategy.

REFERENCES

1. Yu, H., and Rohan, T. Role of the insulin-like growth factor family in cancer development and progression. J. Natl. Cancer Inst. (Bethesda), *92:* 1472–1489, 2000.
2. Sachdev, D., and Yee, D. The IGF system and breast cancer. Endocr. Relat. Cancer, *8:* 197–209, 2001.
3. Dunn, S. E., Hardman, R. A., Kari, F. W., and Barrett, J. C. Insulin-like growth factor 1 (IGF-1) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. Cancer Res., *57:* 2687–2693, 1997.
4. Gooch, J. L., Van Den Berg, C. L., and Yee, D. Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy-induced cell death: proliferative and anti-apoptotic effects. Breast Cancer Res. Treat., *56:* 1–10, 1999.
5. Turner, B. C., Haffty, B. G., Narayanan, L., Yuan, J., Havre, P. A., Gumbs, A. A., Kaplan, L., Burgaud, J. L., Carter, D., Baserga, R., and Glazer, P. M. Insulin-like growth factor I receptor overexpression mediates cellular radioresistance and local breast cancer recurrence after lumpectomy and radiation. Cancer Res., *57:* 3079–3083, 1997.
6. Baserga, R., Sell, C., Porcu, P., and Rubini, M. The role of the IGF-I receptor in the growth and transformation of mammalian cells. Cell Prolif., *27:* 63–71, 1994.
7. Resnik, J. L., Reichart, D. B., Huey, K., Webster, N. J., and Seely, B. L. Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. Cancer Res., *58:* 1159–1164, 1998.
8. Baserga, R. Controlling IGF-receptor function: a possible strategy for tumor therapy. Trends Biotechnol., *14:* 150–152, 1996.
9. Hudziak, R. M., Lewis, G. D., Winget, M., Fendly, B. M., Shepard, H. M., and Ullrich, A. p185HER2 monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol., *9:* 1165–1172, 1989.

[4] J. Ye, S. Liang, N. Guo, S. Li, D. Sachdev, D. Yee, N. Brünner, D. Ikle, and Y. Fujita-Yamaguchi. Combined effects of anti-IFG-I receptor single chain antibody, scFv-Fc, and tamoxifen on breast tumor growth *in vivo*, manuscript in preparation.

10. Fendly, B. M., Winget, M., Hudziak, R. M., Lipari, M. T., Napier, M. A., and Ullrich, A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res., *50:* 1550–1558, 1990.
11. Shak, S. Overview of the trastuzumab (Herceptin) anti-HER2 monoclonal antibody clinical program in HER2-overexpressing metastatic breast cancer. Herceptin Multinational Investigator Study Group. Semin. Oncol., *26:* 71–77, 1999.
12. Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., and Sawyers, C. L. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N. Engl. J. Med., *344:* 1031–1037, 2001.
13. Mauro, M. J., and Druker, B. J. STI571: a gene product-targeted therapy for leukemia. Curr. Oncol. Rep., *3:* 223–227, 2001.
14. Demetri, G. D. Targeting c-kit mutations in solid tumors: scientific rationale and novel therapeutic options. Semin. Oncol., *28:* 19–26, 2001.
15. Andrews, D. W., Resnicoff, M., Flanders, A. E., Kenyon, L., Curtis, M., Merli, G., Baserga, R., Iliakis, G., and Aiken, R. D. Results of a pilot study involving the use of an antisense oligodeoxynucleotide directed against the insulin-like growth factor type I receptor in malignant astrocytomas. J. Clin. Oncol., *19:* 2189–2200, 2001.
16. Parrizas, M., Gazit, A., Levitzki, A., Wertheimer, E., and LeRoith, D. Specific inhibition of insulin-like growth factor-1 and insulin receptor tyrosine kinase activity and biological function by tyrphostins. Endocrinology, *138:* 1427–1433, 1997.
17. Blum, G., Gazit, A., and Levitzki, A. Substrate competitive inhibitors of IGF-1 receptor kinase. Biochemistry, *39:* 15705–15712, 2000.
18. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science (Wash. DC), *237:* 178–182, 1987.
19. Hudziak, R. M., Schlessinger, J., and Ullrich, A. Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA, *84:* 7159–7163, 1987.
20. Kull, F. C., Jr., Jacobs, S., Su, Y. F., Svoboda, M. E., Van Wyk, J. J., and Cuatrecasas, P. Monoclonal antibodies to receptors for insulin and somatomedin-C. J. Biol. Chem., *258:* 6561–6566, 1983.
21. Arteaga, C. L., and Osborne, C. K. Growth inhibition of human breast cancer cells *in vitro* with an antibody against the type I somatomedin receptor. Cancer Res., *49:* 6237–6241, 1989.
22. Rohlik, Q. T., Adams, D., Kull, F. C., Jr., and Jacobs, S. An antibody to the receptor for insulin-like growth factor I inhibits the growth of MCF-7 cells in tissue culture. Biochem. Biophys. Res. Commun., *149:* 276–281, 1987.
23. Brunner, N., Yee, D., Kern, F. G., Spang-Thomsen, M., Lippman, M. E., and Cullen, K. J. Effect of endocrine therapy on growth of T61 human breast cancer xenografts is directly correlated to a specific down-regulation of insulin-like growth factor II (IGF-II). Eur. J. Cancer, *4:* 562–569, 1993.
24. Arteaga, C. L., Kitten, L. J., Coronado, E. B., Jacobs, S., Kull, F. C., Jr., Allred, D. C., and Osborne, C. K. Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. J. Clin. Investig., *84:* 1418–1423, 1989.
25. Li, S. L., Liang, S. J., Guo, N., Wu, A. M., and Fujita-Yamaguchi, Y. Single-chain antibodies against human insulin-like growth factor I receptor: expression, purification, and effect on tumor growth. Cancer Immunol. Immunother., *49:* 243–252, 2000.
26. Li, S. L., Kato, J., Paz, I. B., Kasuya, J., and Fujita-Yamaguchi, Y. Two new monoclonal antibodies against the alpha subunit of the human insulin-like growth factor I receptor. Biochem. Biophys. Res. Commun., *196:* 92–98, 1993.
27. Jackson, J. G., White, M. F., and Yee, D. Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells. J. Biol. Chem., *273:* 9994–10003, 1998.
28. Figueroa, J. A., Sharma, J., Jackson, J. G., McDermott, M. J., Hilsenbeck, S. G., and Yee, D. Recombinant insulin-like growth factor binding protein-1 inhibits IGF-I, serum, and estrogen-dependent growth of MCF-7 human breast cancer cells. J. Cell Physiol., *157:* 229–236, 1993.
29. Lammers, R., Gray, A., Schlessinger, J., and Ullrich, A. Differential signaling potential of insulin- and IGF-1-receptor cytoplasmic domains. EMBO J., *8:* 1369–1375, 1989.
30. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
31. Twentyman, P. R., and Luscombe, M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br. J. Cancer, *56:* 279–285, 1987.
32. McGuire, W. L., Jr., Jackson, J. G., Figueroa, J. A., Shimasaki, S., Powell, D. R., and Yee, D. Regulation of insulin-like growth factor-binding protein (IGFBP) expression by breast cancer cells: use of IGFBP-1 as an inhibitor of insulin-like growth factor action [published erratum appears in J. Natl. Cancer Inst. (Bethesda), *84:* 1837, 1992], J. Natl. Cancer Inst. (Bethesda), *84:* 1336–1341, 1992.
33. Dufourny, B., Alblas, J., van Teeffelen, H. A., van Schaik, F. M., van der Burg, B., Steenbergh, P. H., and Sussenbach, J. S. Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. J. Biol. Chem., *272:* 31163–31171, 1997.
34. Lewis, G. D., Figari, I., Fendly, B., Wong, W. L., Carter, P., Gorman, C., and Shepard, H. M. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol. Immunother., *37:* 255–263, 1993.
35. Lee, A. V., Gooch, J. L., Oesterreich, S., Guler, R. L., and Yee, D. Insulin-like growth factor I-induced degradation of insulin receptor substrate 1 is mediated by the 26S proteasome and blocked by phosphatidylinositol 3′-kinase inhibition. Mol. Cell. Biol., *20:* 1489–1496, 2000.
36. Clynes, R. A., Towers, T. L., Presta, L. G., and Ravetch, J. V. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. Nat. Med., *6:* 443–446, 2000.
37. Clynes, R., Takechi, Y., Moroi, Y., Houghton, A., and Ravetch, J. V. Fc receptors are required in passive and active immunity to melanoma. Proc. Natl. Acad. Sci. USA, *95:* 652–656, 1998.
38. Clarke, R. Human breast cancer cell line xenografts as models of breast cancer. The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. Breast Cancer Res. Treat., *39:* 69–86, 1996.
39. Lopes, U. G., Erhardt, P., Yao, R., and Cooper, G. M. p53-dependent induction of apoptosis by proteasome inhibitors. J. Biol. Chem., *272:* 12893–12896, 1997.
40. Mellman, I., Fuchs, R., and Helenius, A. Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem., *55:* 663–700, 1986.
41. D'Ambrosio, C., Ferber, A., Resnicoff, M., and Baserga, R. A soluble insulin-like growth factor I receptor that induces apoptosis of tumor cells *in vivo* and inhibits tumorigenesis. Cancer Res., *56:* 4013–4020, 1996.
42. Dunn, S. E., Ehrlich, M., Sharp, N. J., Reiss, K., Solomon, G., Hawkins, R., Baserga, R., and Barrett, J. C. A dominant negative mutant of the insulin-like growth factor I receptor inhibits the adhesion, invasion, and metastasis of breast cancer. Cancer Res., *58:* 3353–3361, 1998.
43. Liu, X., Turbyville, T., Fritz, A., and Whitesell, L. Inhibition of insulin-like growth factor I receptor expression in neuroblastoma cells induces the regression of established tumors in mice. Cancer Res., *58:* 5432–5438, 1998.
44. Ciardiello, F., and Tortora, G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin. Cancer Res., *7:* 2958–2970, 2001.
45. Albanell, J., Rojo, F., and Baselga, J. Pharmacodynamic studies with the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839. Semin. Oncol., *28:* 56–66, 2001.
46. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., *et al.* Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J., *5:* 2503–2512, 1986.
47. Pautsch, A., Zoephel, A., Ahorn, H., Spevak, W., Hauptmann, R., and Nar, H. Crystal structure of bisphosphorylated IGF-1 receptor kinase: insight into domain movements upon kinase activation. Structure (Camb.), *9:* 955–965, 2001.
48. Herbst, R. S., and Langer, C. J. Epidermal growth factor receptors as a target for cancer treatment: the emerging role of IMC-C225 in the treatment of lung and head and neck cancers. Semin. Oncol., *29:* 27–36, 2002.
49. Xiong, L., Kasuya, J., Li, S. L., Kato, J., and Fujita-Yamaguchi, Y. Growth-stimulatory monoclonal antibodies against human insulin-like growth factor I receptor. Proc. Natl. Acad. Sci. USA, *89:* 5356–5360, 1992.
50. Xu, F. J., Boyer, C. M., Bae, D. S., Wu, S., Greenwald, M., O'Briant, K., Yu, Y. H., Mills, G. B., and Bast, R. C., Jr. The tyrosine kinase activity of the C-erbB-2 gene product (p185) is required for growth inhibition by anti-p185 antibodies but not for the cytotoxicity of an anti-p185-ricin-A chain immunotoxin. Int. J. Cancer, *59:* 242–247, 1994.
51. Shawver, L. K., Mann, E., Elliger, S. S., Dugger, T. C., and Arteaga, C. L. Ligand-like effects induced by anti-c-erbB-2 antibodies do not correlate with and are not required for growth inhibition of human carcinoma cells. Cancer Res., *54:* 1367–1373, 1994.
52. Stancovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y., and Sela, M. Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. Proc. Natl. Acad. Sci. USA, *88:* 8691–8695, 1991.
53. Furlanetto, R. W. Receptor-mediated endocytosis and lysosomal processing of insulin-like growth factor I by mitogenically responsive cells. Endocrinology, *122:* 2044–2053, 1988.
54. Hsu, D., Knudson, P. E., Zapf, A., Rolband, G. C., and Olefsky, J. M. NPXY motif in the insulin-like growth factor I receptor is required for efficient ligand-mediated receptor internalization and biological signaling. Endocrinology, *134:* 744–750, 1994.
55. Zapf, A., Hsu, D., and Olefsky, J. M. Comparison of the intracellular itineraries of insulin-like growth factor I and insulin and their receptors in Rat-1 fibroblasts. Endocrinology, *134:* 2445–2452, 1994.
56. Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A., and Greene, M. I. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell, *41:* 697–706, 1985.
57. Leonessa, F., Green, D., Licht, T., Wright, A., Wingate-Legette, K., Lippman, J., Gottesman, M. M., and Clarke, R. MDA435/LCC6 and MDA435/LCC6MDR1: ascites models of human breast cancer. Br. J. Cancer, *73:* 154–161, 1996.
58. Maier, L. A., Xu, F. J., Hester, S., Boyer, C. M., McKenzie, S., Bruskin, A. M., Argon, Y., and Bast, R. C., Jr. Requirements for the internalization of a murine monoclonal antibody directed against the HER-2/neu gene product c-erbB-2. Cancer Res., *51:* 5361–5369, 1991.
59. Brunner, N., Bastert, G. B., Poulsen, H. S., Spang-Thomsen, M., Engelholm, S. A., Vindelov, L., Nielsen, A., Tommerup, N., and Elling, F. Characterization of the T61 human breast carcinoma established in nude mice. Eur. J. Cancer Clin. Oncol., *21:* 833–843, 1985.
60. Brunner, N., Spang-Thomsen, M., and Cullen, K. The T61 human breast cancer xenograft: an experimental model of estrogen therapy of breast cancer. Breast Cancer Res. Treat., *39:* 87–92, 1996.
61. Reiss, K., Valentinis, B., Tu, X., Xu, S. Q., and Baserga, R. Molecular markers of IGF-I-mediated mitogenesis. Exp. Cell Res., *242:* 361–372, 1998.
62. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D., and Pollak, M. Insulin-like growth factor I receptor signaling and resistance to trastuzumab (Herceptin). J. Natl. Cancer Inst. (Bethesda), *93:* 1852–1857, 2001.
63. Chakravarti, A., Loeffler, J. S., and Dyson, N. J. Insulin-like growth factor receptor I mediates resistance to antiepidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. Cancer Res., *62:* 200–207, 2002.

A dominant negative type I insulin-like growth factor-1 receptor inhibits metastasis of human cancer cells.

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Running title: Role of the type I IGF receptor in metastasis

* This work was supported by USAMRMC Grant DAMD17-00-1-0347 (to D.S.), NIH Grant R01 CA74285 and PHS Cancer Center Support Grant P30 CA77398.

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SUMMARY

We have previously shown that LCC6-WT cells, a metastatic variant of MDA-MB-435 cancer cells originally derived from a breast cancer patient, exhibit enhanced motility in response to IGF-I compared to the parent MDA-MB-435 cells. To further understand the role of the type I IGF receptor (IGF1R) in cancer metastasis, we inhibited signaling via IGF1R using a C-terminally truncated IGF1R. The truncated receptor retains the ligand binding domain but lacks the autophosphorylated tyrosine residues in the carboxy-terminus. Cells stably transfected with this truncated receptor (LCC6-DN cells) overexpressed the truncated IGF1R messenger RNA nearly 50-fold over endogenous receptor. The truncated receptor in the LCC6-DN cells behaved in a dominant negative manner to inhibit endogenous IGF1R activation by IGF-I. Compared to the LCC6-WT cells, LCC6-DN cells failed to phosphorylate the adaptor proteins insulin receptor substrate-1 and -2 (IRS-1/2) in response to IGF-I and did not activate the phosphatidyl inositol 3' kinase (PI3K) pathway after exposure to IGF-I. Unlike LCC6-WT cells, LCC6-DN cells did not show enhanced motility in response to IGF-I. To assay for metastasis, LCC6-WT and LCC6-DN cells were injected into the mammary fat pads of mice and the primary xenograft tumors were removed after 21 days. Mice sacrificed five weeks later showed multiple lung metastases derived from LCC-WT xenografts while mice harboring LCC6-DN xenografts showed no lung metastases. Our data show that IGF1R can regulate several aspects of the malignant phenotype. In these cells, metastasis, but not proliferation, requires IGF1R function.

INTRODUCTION

Insulin-like growth factors (IGFs) have pleiotropic effects on normal and cancer cells. These effects are mediated by the type I insulin-like growth factor receptor (IGF1R). Several lines of evidence suggest that the IGF system and IGF1R are relevant to the malignant transformation of cells. Overexpression of IGF1R causes ligand dependent transformation of fibroblasts (1). Embryonic fibroblasts obtained from IGF1R knockout mice cannot easily be transformed by simian virus 40 large tumor antigen (SV40T) or activated *ras* (2). When these fibroblasts are then stably transfected with IGF1R, they can be transformed by SV40T suggesting that a functional IGF1R is required for oncogenic transformation (3). IGF1R may be required for oncogenic transformation and tumorigenicity of cells (4). The IGF system has also been implicated in maintaining the malignant phenotype. Mice with low circulating levels of IGF-I have reduced incidence of colon tumor growth and metastasis of the colon adenocarcinoma to the liver (5). When IGF-I is expressed in mammary gland of mice, these animals have increased rates of developing breast cancer (6). Elevated levels of IGF-I are associated with increased risk of developing breast, prostate and colon cancer (7,8).

IGF1R has been reported to be involved in several different cancers including breast cancer, prostate cancer, liver cancer, colon cancer, melanoma, glioblastomas, Ewing's sarcoma (9) and childhood malignancies. Activation of this receptor has been reported to be enhanced in breast cancer (10). IGF1R protects cancer cells from chemotherapy (11-13), causes radioresistance (14) and enhances proliferation (15,16). Blocking antibodies that inhibit binding of IGF-I to IGF1R inhibit tumor growth in mice

(17,18) demonstrating the importance of this receptor in tumorigenesis. IGF-I has also been shown to enhance adhesion and motility of several cancer cell types (19-25). However, the role of IGF1R in the metastatic process is not fully defined.

IGF1R consists of two covalently linked polypeptide chains each with an extracellular α-subunit that binds ligand and a transmembrane β-subunit which contains tyrosine kinase activity. The IGF1R is transported to the membrane fully assembled in the dimeric form and after ligand binding, tyrosine kinase activity is initiated. Binding of ligand results in transphosphorylation of the β-subunit of one chain by the opposite β-subunit chain. This transphosphorylation is required for activation of the receptor and activation of downstream signaling pathways (26). Therefore, to study the role of the IGF1R in the metastasis of cancer cells, we have overexpressed a C-terminally truncated IGF1R that retains the ligand binding domain but lacks the autophosphorylated tyrosine residues in the carboxy-terminus. When the protein is assembled with either wild-type or truncated receptor construct, such a receptor can bind ligand but not be transphosphorylated.

It has been shown that a truncated IGF1R when expressed in rat fibroblasts causes inhibition of tumorigenesis (27). Dunn *et al.*, 1998 (20) have used the extracellular ligand binding domain of IGF1R as a soluble receptor to neutralize circulating IGFs. This soluble receptor has been reported to behave in a dominant negative manner (28) and inhibited the adhesion, motility and metastasis of MDA-MB-435 cells (20). In this approach, it was possible that these effects were due to inhibition of circulating IGF

effects on either host or tumor tissue. To directly study the effect of inhibiting IGF1R in the cancer cells and its impact on metastasis, we used the MDA 435A/LCC6 cells (LCC6-WT) (29), which are a metastatic variant of the ER-negative MDA-MB-435 cells (30) derived from a patient with breast cancer. Recent reports suggest that the MDA-MB-435 cells have a gene expression pattern consistent with malignant melanomas (31,32).

We have previously shown that the LCC6-WT cells exhibit enhanced motility in response to IGF compared to the parent MDA-MB-435 cells (33). To examine the role of IGF1R in the metastasis of breast cancer cells we transfected the C-terminally truncated IGF1R into the LCC6-WT cells. We found that LCC6 cells stably transfected with a truncated IGF1R (LCC6-DN) failed to activate IGF1R and downstream signaling pathways in response to IGF-I. LCC6-DN cells were unable to metastasize to the lungs in a xenograft model of tumor growth. These results suggest that IGF1R, in addition to its well-known role in stimulating proliferation of breast cancer cells, plays an essential role in the metastasis of these cancer cells. Moreover, these metastatic events may be regulated independently of proliferative signals. Thus, targeting IGF1R function can affect the metastasis of cancer cells and anti-IGF therapy may inhibit several characteristics of the malignant phenotype.

EXPERIMENTAL PROCEDURES

Reagents. All reagents and chemicals were purchased from Sigma (St. Louis, MO) and cell culture reagents were from Invitrogen/Life Technologies (Rockville, MD) unless otherwise noted. IGF-I was purchased from Gro*Pep* (Adelaide, Australia). Anti-phosphotyrosine antibody (PY-20) conjugated to horseradish-peroxidase (HRP) was from BD Transduction Laboratories (Lexington, KY). Antibodies against ERK1/ERK2 (phosphospecific and total) and Akt (phosphospecific and total) were purchased from Cell Signaling (Beverly, MA). The rabbit polyclonal antibody against IRS-1 was produced by Alpha Diagnostics (San Antonio, TX) and the immune serum was affinity purified on immobilized protein A as described previously (34). The antibody against IRS-2 was purchased from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibody against the β-subunit of IGF1R (IGF1Rβ) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the polyclonal antibody against hemagluttinin A (HA) was from Covance (Berkeley, CA). Anti-rabbit secondary antibody conjugated to HRP was from Amersham Biosciences (Piscataway, NJ). Protein A-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acrylamide, bis-acrylamide and prestained molecular weight markers were from Bio-Rad (Hercules, CA).

Cell lines and culture. LCC6-WT cells were obtained from Dr. Robert Clarke at the Lombardi Cancer Center, Georgetown University, Washington D.C. LCC6-WT and LCC6-DN cells were routinely maintained in Dulbecco's modified Eagle's medium

(DMEM) with 10% fetal bovine serum (FBS), 11.25 nM human insulin (Eli Lilly, Indianapolis, IN), 50 U/ml penicillin and 50 μg/ml streptomycin.

LCC6 cells were transfected with a CMV-promoter driven expression vector encoding IGF1R truncated at codon 2929 tagged with the influenza hemagluttinin A epitope. The vector was a kind gift from Dr. Mikhail L. Gishizky at Sugen Inc., (San Francisco, CA). G418 resistant colonies were selected, expanded and screened for expression of truncated IGF1R by ribonuclease (RNAse) protection assay. LCC6 cells expressing the truncated IGF1R are referred to as LCC6-DN.

RNAse protection assay. RNA from cells was isolated by the guanidinium thiocyanate method (35). 10 μg of a plasmid encoding IGF1R was linearized and used as riboprobe in RPA. RPA was performed with a probe encompassing codons 2737 to 3195 as previously described (36).

Cell stimulation. LCC6-WT and LCC6-DN cells were grown in 10 cm dishes in regular growth media. 70% confluent cells were washed two times with phosphate-buffered saline (PBS) and serum deprived for 24 h in serum free media (SFM) as described previously (37). For treatment with IGF-I, medium was replaced with SFM containing 5 nM IGF-I for ten minutes.

Cell lysis. Cells were washed three times with ice-cold PBS on ice and lysed with 500 μl/10 cm dish lysis buffer TNESV (50 mM Tris-Cl, pH 7.4; 1% NP-40; 2 mM EDTA, pH 8.0; 100 mM NaCl, 10 mM Na orthovanadate, 1 mM PMSF, 20 μg/ml

leupeptin, and 20 μg/ml aprotinin). Lysates were clarified by centrifugation at 12000g for 20 minutes at 4°C and soluble cellular proteins were used for experiments or stored at –20°C. Protein concentrations of the lysates were determined using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL).

Immunoblotting. For immunoblotting, 40 μg of cellular proteins were subjected to reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% polyacrylamide gels following the Laemmli system (38). After SDS-PAGE, proteins were transferred to nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST – 0.15 M NaCl; 0.01 M Tris-HCl pH 7.4; 0.05% Tween-20) for 1 h at room temperature with gentle shaking. Membranes were then washed five times with TBST for 5 minutes each. Expression of truncated IGF1R in LCC6-DN cells was assayed by blotting with 1:500 dilution of HA antibody for 1 h at room temperature. To detect levels of IGF1R membranes were blotted with 1:1000 dilution of rabbit polyclonal antibody against IGF1Rβ. Membranes were washed five times with TBST for 5 minutes each and incubated with 1:2000 dilution of anti-rabbit secondary antibody conjugated to HRP for 1 h at room temperature. Membranes were washed as before and chemiluminescence was done using SuperSignal West Pico substrate (Pierce, Rockford, IL).

For detecting phosphorylated proteins, membranes were incubated with 1:10,000 dilution of PY-20 anti-phosphotyrosine antibody in TBST for 1 h at room temperature. Membranes were washed as before. Chemiluminescence was then performed as

plate reader at 570 nm using a 670 nm differential filter. Proliferation assays were repeated 3 times.

Anchorage-independent growth. Anchorage-independent growth assays were performed as described (40). 1 x 10^4 cells per well of a 6-well plate were used. 1 ml of 0.8% SeaPlaque agarose (BioWhitaker, Rockland, ME) in culture medium was solidified in the bottom of each well as the bottom agar. Cells in growth media with 5% serum without or with 5 nM IGF-I were mixed with 0.45% agarose. The cells mixed with agarose were overlaid on the bottom agar. After 9-10 days, colonies were counted using a microscope with a grid in the eyepiece. Three randomly selected fields were counted for each well and the average number of colonies is shown. Results shown are representative of one experiment with triplicates for each treatment.

Motility assay. Motility was assayed by a modified Boyden chamber assay using 10-well chambers (NeuroProbe Inc., Gaithersburg, MD) with 12 μm pores as described previously (33). 0.4 ml of SFM with or without 5 nM IGF-I was placed in the lower wells of the chamber. A polycarbonate polyvinylpyrrolidine-free filter was placed above the lower wells. Cells were quickly trypsinized and washed twice with PBS. Cells were suspended in SFM and 1.5 x 10^5 cells in 0.3 ml of SFM were placed in each of the upper wells of the chamber above the membrane. Cells were allowed to migrate for 4 h at 37°C. Cells still remaining on the upper side of the membrane were removed with a cotton-tipped applicator. The membrane was removed and cells that had migrated to the lower side of the membrane were fixed and stained with HEMA3 (Fisher Scientific). The

membrane was then mounted on a glass slide and cells were counted in 10 random areas using a microscope. The assay was repeated 3 independent times.

Tumor growth in athymic mice. 4-week old female athymic mice were used for xenograft tumor growth. 5 x 10^6 LCC6-WT or LCC6-DN cells in serum-free IMEM were injected into the mammary fat pad on one side of each mouse. Five mice were injected with LCC6-WT and five with LCC6-DN cells. Tumor growth was measured weekly and is shown as average tumor volume for each cell line with a n=5. Tumor growth experiments were repeated three times. At the end of the experiment, mice were sacrificed and the tumors were snap frozen in liquid nitrogen. Frozen tumor samples were homogenized in a tissue pulverizer in a dry ice/ethanol bath. Tissue homogenates were suspended in 500 µl lysis buffer, TNESV.

Analysis of tumor metastasis. All mice injected with each cell line were sacrificed at day 21-24, the whole animal was fixed in 4% neutral buffered formalin, and analyzed for metastasis by gross and microscopic examination. Lungs fixed in 4% neutral buffered formalin were examined macroscopically for metastases. Fixed lungs were also embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically for metastatic deposits. Slides prepared from the lungs included a complete longitudinal section from the left lobe, and several transverse sections through the right lobe.

To confirm the presence of metastases, primary xenograft tumors were resected from 2 mice in each group at day 21 after injecting cells and mice were allowed to

recover. At day 54, of the 10 mice in the experiment, two mice injected with LCC6-WT and two with LCC6-DN cells, whose tumors had been resected at day 21, were sacrificed and dissected. The whole animal was fixed in 4% neutral buffered formalin. All organs were examined for metastasis by gross and microscopic examination.

RESULTS

Expression of C-terminally truncated IGF1R in LCC6 cells. LCC6 cells were stably transfected with the construct for truncated IGF1R. Expression of the truncated IGF1R was measured by RNAse protection assay (Fig. 1). The probe covers the full length transcript (458 bp) and also extends over a natural splice site that leads to the alternately spliced transcript of 366 bp (36). Truncated IGF1R protected a fragment of 262 bp. RNA from MCF-7 cells was used as control and the full-length 458 bp protected fragment and the alternately spliced variant fragment of 366 bp were detected (lane 2). Similarly, the LCC6-WT cells also expressed the full-length 458 bp and alternately spliced 366 bp fragments as shown in lane 3. Neither MCF-7 nor LCC6-WT expressed the message for the truncated receptor. Individual LCC6-DN clones expressed the message for the full-length fragments as shown in lanes 4-8. In addition, several clones overexpressed the message for the truncated receptor (262 bp protected fragment in lanes 4-8). Several clones expressing the message for the truncated were selected for further study. All of the clones behaved in a similar manner to inhibit activation of endogenous IGF1R (Fig. 3B) and a single clone was selected for further characterization.

In this study, we used clone number 6 (in lane 5, Fig. 1) and it is referred to as LCC6-DN cell line. The levels of the truncated protein were assayed by HA immunoblotting as shown in Fig. 2, left panel. In lane 1, LCC6-WT cells did not express the truncated HA-tagged IGF1R while in lane 2, LCC6-DN cells expressed the HA-tagged truncated IGF1R at 50 kDa. Some of the higher molecular weight unprocessed

receptors were also detected in lane 2. Levels of endogenous full-length receptor were detected using an antibody directed against the C-terminus of IGF1Rβ. Figure 2, right panel shows that levels of endogenous IGF1R between LCC6-WT and LCC6-DN cells were similar.

Truncated IGF1R behaved in a dominant negative manner to inhibit IGF1R activation by IGF-I. In LCC6-WT cells we have previously shown that IRS-2 is the major adaptor protein phosphorylated by IGF-I mediated activation of IGF1R (33). As reported previously (37) and shown in Fig. 3A, IGF-I treatment of LCC6-WT cells resulted in detection of a 185 kDa phosphorylated band in IGF-I treated cells (lane 2) but not in untreated cells (lane 1). In contrast in LCC6-DN cells, IGF-I treatment did not result in significantly enhanced phosphorylation of the 185 kDa protein (lane 4 compared to lane 3). As a control, we used MCF-7 cells which phosphorylated IRS-1 in response to IGF-I (lanes 5 and 6). All of the other clones expressing truncated IGF1R showed similar inhibition of IGFR activation, as shown in Fig. 3B. All of the *in vitro* studies on signaling via the IGF1R described below were done on four independent clones and they all behaved similarly (data not shown).

To ensure that LCC6-DN had reduced phosphorylation of IRS species, we immunoprecipitated lysates with antibodies specific for IRS-1 (Fig. 3C) or IRS-2 (Fig. 3D) followed by anti-phosphotyrosine blotting. As shown in Fig. 3, LCC6-WT phosphorylated both IRS-1 (lane 2, 3C) and IRS-2 (lane 2, 3D) after IGF-I treatment. LCC6-DN cells, however, did not phosphorylate IRS-1 in response to IGF-I (Fig. 3C,

lane 4 compared to 3). They also had much less phosphorylation of IRS-2 (Fig. 3D, lane 4 compared to 3) after IGF treatment, compared to LCC6-WT cells. As controls for the immunoprecipitations, we used MCF-7 cells (Fig. 3C, lane 6 compared to lane 5) for IRS-1 and MDA-231BO cells for IRS-2 (Fig. 3D, lane 6 compared to lane 5). We have previously shown that in MCF-7 cells, IRS-1 is the major species activated by IGF-I treatment (37) and in MDA-231BO cells IRS-2 is the major protein phosphorylated by IGF-I treatment (33). These results indicate that 50-fold overexpression of truncated IGF1R in LCC6-DN cells resulted in markedly diminished activation of endogenous full-length IGF1R in these cells.

Dominant negative IGF1R inhibited downstream signaling pathways. We next examined signaling pathways distal to IRS. We and others have shown that activation of IGF1R results in phosphorylation of p44/p42 extracellular signal-regulated protein kinase (ERK1/ERK2) of the MAPK pathway (37) and activation of the PI3K pathway (37,41). LCC6-WT and LCC6-DN cells were treated with or without IGF-I and phosphorylation of Akt, a substrate for PI3K, was measured (Fig. 4A). In LCC6-WT cells, IGF-I treatment activated the PI3K pathway as shown by the phosphorylation of Akt in lane 4 versus lane 3 in Fig. 4A. In LCC6-DN cells, phosphorylation of Akt was not detected following IGF-I treatment (lane 6 versus lane 5). As controls we used MDA-231BO (lanes 1 and 2) and MCF-7 cells (lanes 7 and 8) which we have previously shown activated the PI3K pathway in response to IGF-I (37). Figure 4B shows total levels of Akt were similar among the cell lines.

We next examined activation of ERK1/ERK2 using a phosphospecific p44/p42 antibody. In both LCC6-WT and LCC6-DN cells, p44/p42 ERK1/ERK2 was constitutively phosphorylated (Fig. 4C, lanes 3 and 5) and IGF-I did not cause any further phosphorylation in LCC6-WT (lane 4) or LCC6-DN (lane 6). Constitutive phosphorylation of ERK1/ERK2 in MDA-231BO was also seen (Fig. 4B, lanes 1 and 2) as previously shown. As control, we show MCF-7 cells in which IGF-I treatment resulted in phosphorylation of ERK1/ERK2 (lane 8 vs. lane 7). The total levels of ERK1/ERK2 (Fig. 4D) were equivalent among cells.

Dominant negative IGF1R did not affect proliferative responses to IGF-I or serum in vitro. We and others have shown that IGF-I does not stimulate the proliferation of ER- cells such as MDA-MB-231 cells. As shown in Fig. 5, IGF-I did not stimulate the growth of LCC6-WT cells, yet these cells responded to serum. LCC6-DN cells behaved in a similar fashion. Thus, dominant negative IGF1R did not have an effect on the growth of these cells *in vitro* as LCC6-DN proliferated equally well to serum suggesting that IGF1R was not associated with a proliferative response in these cells.

IGF-I did not stimulate motility of LCC6-DN cells. We have previously reported that LCC6-WT cells showed greater motility in response to IGF-I compared to their parental MDA-MB-435 cells (33). Therefore, we measured the effect of the truncated IGF1R on motility of LCC-DN cells in a modified Boyden chamber assay. As shown in Fig. 6, LCC6-WT and MDA-231BO cells had increased motility in response to IGF-I. In

contrast, IGF-I did not enhance the motility of LCC6-DN cells. However, basal motility of these cells was greater than that of LCC6-WT cells.

LCC6-DN cells had decreased anchorage independent growth in response to IGF-I. In order to form tumors, cancer cells need to grow in an anchorage-independent manner. Therefore, to measure the metastatic potential of these cells, we compared their ability to grow in an anchorage-independent soft agar assay. LCC6-WT cells formed colonies in soft agar as shown in Fig. 7 and IGF-I increased colony formation in soft agar. Compared to LCC6-WT, LCC6-DN cells formed fewer colonies and IGF-I did not increase colony formation by LCC6-DN cells.

Xenograft tumor growth was similar in cells with wild-type IGF1R and dominant-negative IGF1R. To determine if dominant negative IGF1R inhibits tumor growth, LCC6-WT and LCC6-DN cells were injected into the mammary fat pad of athymic mice. Both LCC6-WT and LCC6-DN cells formed xenograft tumors in athymic mice and no significant differences in tumor volumes between LCC6-WT and LCC6-DN tumors were detected in two independent experiments (data not shown). When the experiment was repeated a third time as shown in Fig. 8, the LCC6-DN tumors were marginally smaller in size (average tumor volume at day 21 of 924.4 mm^3, n=5) than the LCC6-WT tumors (tumor volume of 1400 mm^3, n=5). When tumors from the mice in each group were removed after the mice were sacrificed and analyzed for expression of the truncated IGF1R by immunoblotting for HA, all tumors from LCC6-DN group still expressed the truncated receptor (data not shown).

Histologically, mammary fat pad tumors in mice injected with LCC6-WT or LCC6-DN cells were composed of densely packed sheets of round to oval, pleomorphic cells with varying amounts of eosinophilic cytoplasm and indistinct cell borders (data not shown). The nuclei were ovoid and had 1 to 5 distinct nucleoli. The mitotic index was high in both LCC6-WT and LCC6-DN tumors suggesting that they were equally aggressive locally.

IGF1R dominant negative cells did not metastasize to lungs in athymic mice. As MDA-MB-435 and LCC6 cells have been reported to metastasize to lungs (42,43), we examined the lungs for metastatic deposits to determine the effect of inhibition of IGF1R on this process. When mice were sacrificed at the end of the first two experiments (day 21-24), all mice (5/5) with LCC6-WT xenograft tumors showed numerous pulmonary micrometastases while 0/5 mice with LCC6-DN xenograft tumors showed metastases. To further explore this finding, we surgically resected the primary tumors from two mice in each group at day 21 and then sacrificed these mice at day 54. When these mice were then examined for metastatic deposits, we found that both mice with LCC6-WT xenograft tumors had gross lung metastases while the 2 mice with LCC6-DN xenograft tumors did not. Both mice with LCC6-WT tumors had numerous well circumscribed lung metastases. Figure 9 shows a heart and lung block removed from an animal harboring LCC6-WT tumor (left panel). Histomorphologically, the pulmonary metastases were similar to the primary tumor origin in the mammary fat pad (data not shown). In contrast, a lung from a mouse with LCC6-DN xenograft is shown in the right

panel and showed no metastases. No gross metastases were seen in the lungs of all animals with LCC6-DN cells, although two micrometastatic cell clusters were found in the right lung of one mouse with LCC6-DN xenograft tumor at day 54. Thus, dominant negative IGF1R inhibited formation of lung metastases in athymic mice suggesting that IGF1R may be essential for the metastasis of these cells.

DISCUSSION

Cancer is often an incurable disease because many patients already have metastatic spread of their disease at first diagnosis. Recently there has been a plethora of reports on the metastatic process and a better understanding of this process is emerging (44). The process of metastasis involves the ability of cancer cells to invade the basement membrane of cells, intravasate the blood vessels and enter circulation, survive in the circulation to reach distant sites, extravasate from vessels into the surrounding tissue at the new site, and finally arrest in the metastatic sites and colonize them (45-47). While it is clear that extracellular proteins involved in cell adhesion play a role in metastasis, it is not clear if theses processes can be regulated by peptide growth factors.

The IGF system has been implicated in various characteristics associated with the malignant phenotype. IGFs promote the proliferation, survival, motility and metastasis of cancer cells. The role of IGF1R in stimulating proliferation of tumor cells is well-defined and inhibition of IGF1R activation using an inhibitory antibody against IGF1R such as αIR3 has been shown to inhibit xenograft growth of cancer cells (17,18). However, the role of IGF1R in the metastasis of breast cancer is incompletely defined even though this system has been implicated in the metastasis of several cancers.

IGFs and IGF1R have been shown to influence the metastasis of several cancers. Low circulating levels of IGF-I in liver-specific IGF-I deficient (LID) mice has been related to decreased metastasis of gastric and colon cancer in these mice (5). Using a mouse model of pancreatic islet cell tumorigenesis, it has been shown that IGF1R is

upregulated focally at the margins and in invasive regions of carcinomas (48). Gene array analyses have shown that IGF1R is significantly increased in a variant of a mouse breast cancer cell line that metastasizes to the brain compared to the parent cell line (49). Our laboratory has also shown that IGF1R levels are increased in a variant of MDA-MB-231 breast cancer cells that were selected *in vivo* for metastasis to the bone (33). Furthermore, IGF1R has been implicated in the metastasis of uveal melanomas (50) and lung carcinoma (51). It also promotes invasiveness of pancreatic cancer cells.

We and others have shown that *in vitro* interaction between IGF1R and integrins may be required for motility of cells (52-54). In MDA-231BO cells, which are a variant of the MDA-MB-231 cells selected for increased metastasis to bone *in vivo*, both IGF1R activation and $\alpha 5\beta 1$ integrin occupancy are required for motility (52). It has also been shown that ligand occupancy of $\alpha V\beta 3$ is required for IGF-I stimulated motility of smooth muscle cells (55). It has been suggested that IGF1R, integrins and extracellular matrix may play a coordinated role in IGF-I stimulated motility of cancer cells (19). We were interested in determining the role of IGF1R in the metastasis process and whether IGF1R by itself has an effect on metastasis. Therefore, we inhibited cancer cell IGF1R function by overexpressing a truncated IGF1R that lacks the autophosphorylated residues. We have shown here that this truncated receptor behaved in a dominant negative manner to inhibit activation of endogenous IGF1R by IGF-I (Fig. 3) and also inhibited activation of the downstream PI3K signaling pathway (Fig. 4). In LCC6 and MDA231BO cells, ERK1/2 of the MAPK pathway are constitutively active and IGF did not further enhance the activation. Our results suggest that the MAPK pathway is not involved in the

metastatic processes regulated by IGF-I, as it is basally active in these cells and IGF-I treatment does not result in further activation of this pathway. Other downstream signaling pathways such as RhoGTPases may be involved (Zhang and Yee, personal communication).

Furthermore, we have shown in this study that cells expressing the dominant negative IGF1R had decreased colony formation in agar but lack of functional IGF1R did not affect their proliferation rates *in vitro*. This result was expected in view of the fact that IGFs do not promote proliferation of the parent MDA-MB-435 or the metastatic variant LCC6 cells (Fig. 5). We had previously shown that inhibition of IGF-I action with IGFBP-1 also inhibits anchorage-independent growth of LCC6 cells (56). Since IGFBP-1 interrupts integrin function and IGF1R activation, these experiments with dominant negative IGF1R show that disruption of IGF1R alone can inhibit anchorage-independent growth of these cells. In contrast, LCC6-DN cells did not show increased motility in response to IGF-I. However, we noticed a higher basal motility rate of LCC6-DN cells compared to LCC6-WT cells. We have seen this previously in MDA-231BO cells transfected with an antisense construct to IRS-2 compared to MBA-MB-231BO cells (33). It is possible that cells selected *in vivo* for increased metastasis such as LCC6 and MDA-231BO become less adhesive and IGF-I regulates their adhesion and motility on specific substrates (33). When IGF-I signaling is interrupted in these cells, they become more adhesive and IGF-I does not enhance their motility. This increased adhesion then results in higher basal motility rates as measured by the Boyden chamber assay.

There are other reports of the ability of dominant negative IGF1R to inhibit cancer cell biology. Prager *et al.* (27) transfected a dominant negative IGF1R into rat fibroblasts and showed that it inhibited tumorigenesis. Our studies show that IGF1R is not required for tumorigenesis of LCC6 cells. In contrast IGF1R is required for metastasis. Dunn *et al.* (20) have also reported that a IGF1R construct that behaves in a dominant negative manner inhibits motility and metastasis of MDA-MB-435 cells. Their approach, however, was different from that described in this work. Their construct created a point mutation in the α-subunit resulting in a stop codon in the extracellular domain. This resulted in the secretion of a soluble truncated IGF1R with only the extracellular domain of IGF1R (28). This soluble IGF1R then serves to neutralize circulating ligands. In their approach, it is possible that the effect on metastasis may be due to inhibiting the effects of the host IGF system as this study did not show a direct effect on IGF signaling in the cells expressing the dominant negative construct. Our results clearly show that inhibition of IGF1R signaling within the cancer cell blocks pulmonary metastases.

Recently there have been other reports describing the role of IGF1R in metastasis of human colon cancer. Reinmuth *et al.* have recently shown that IGF1R plays a role in multiple mechanisms that mediate angiogenesis and metastasis of human colon cancer cells (57). Using a dominant negative IGF1R, they have shown following splenic injection, cells expressing dominant negative IGF1R failed to form liver metastases and they also failed to form liver tumors when injected into the livers. Our experiments did

not involve direct injection of tumor cells into the vascular space. Thus, it appears that IGF1R plays an important role in the complete metastatic phenotype, from breaching the basement membrane to establishment of growth in distant sites.

The precise steps in the metastatic cascade that are regulated by IGF1R are not yet clear. However, our data suggest that perhaps IGF1R is required for colonization at metastatic sites. It is possible that these cells cannot live in the metastatic environment without functional IGF1R. Alternately, cells with dominant negative IGF1R could be less invasive suggesting that inhibition of IGF1R function has inhibited the ability of cells to invade through the basement membrane. However, this appears not to be the case because in our experiments both LCC6-WT and LCC6-DN tumors appeared to be equally aggressive locally. As IGF1R has also been implicated in adhesion of cells and cell-cell contact (58,59), it is possible that functional impairment of IGF1R in LCC6-DN cells has inhibited regulated adhesion to cell surfaces. Perhaps impairment of IGF1R function in these cells affected the ability of IGF-I to induce production of cytokines such as IL-8. It has recently been suggested that IGF-I can increase production of IL-8 in melanomas by increasing its transcription rate (60).

As IGF1R targeting strategies are being developed (34,61), understanding the function and signaling pathways will be required to interpret the effects of an anti-IGF agent. In the models of cancer metastasis, it appears that IGF1R can regulate both growth regulatory and metastatic signals. Moreover, response to IGF1R activation may not be easily predicted by levels of receptor expression. Accounting for the differences

in tumor cell biology regulated by IGF1R must be considered when designing clinical studies.

Acknowledgment-We wish to thank Dr. Gishizky at Sugen Inc., for the kind gift of the truncated IGF1R construct and Dr. Nicole Kirchoff at the Histopathology Core of the University of Minnesota Cancer Center for assistance with the histology of the tumors and metastases.

FIGURE LEGENDS

FIG. 1. Expression of IGF1R mRNA in LCC6-DN cells. RNAse protection was used to measure levels of the truncated IGF1R mRNA in LCC6-DN cells. The full-length protected transcript is 458 bp and a naturally occurring splice site transcript is 366 bp as shown in MCF-7 cells (lane 2). LCC6-WT cells (lane 3) also show the 458 and 366 bp fragments. Lanes 4-8 show independent clones selected for expression of truncated IGF1R. 4 out of the 5 clones overexpressed the message for the truncated 262 bp fragment.

FIG. 2. LCC6-DN cells overexpressed truncated IGF1R. 40 μg of cellular proteins from LCC6-WT (lanes labeled WT) and LCC6-DN (lanes labeled DN) cells were subjected to SDS-PAGE. Protein levels of the truncated IGF1R in LCC6-DN cells were assayed by immunoblotting for Hemagluttinin A (left panel). Cell lysates were also immunoblotted for total levels of full length endogenous IGF1Rβ (right panel) using an antibody against the C-terminus of IGF1R. The 97 kDa β-subunit of IGF1R is shown.

FIG. 3. C-terminally truncated IGF1R inhibited activation of endogenous IGF1R. 70% confluent cells were switched to serum-free medium as described in Materials and Methods. 24 h later, cells were treated with 5 nM IGF-I for 10 minutes and cell lysates were examined by immunoblot. **A.** Anti-phosphotyrosine immunoblot. LCC6-WT cells (lanes 1 and 2) and LCC6-DN cells (lanes 3 and 4) were either untreated (lanes 1 and 3) or treated with 5 nM IGF-I (lanes 2 and 4). As controls, lanes 5 and 6 show MCF-7 cells

in which IGF-I treatment results in phosphorylation of IRS-1 (lane 6 vs. 5). **B.** Anti-phosphotyrosine immunoblot on several independent clones overexpressing truncated IGF1R to show that all clones overexpressing truncated IGF1R inhibit activation of endogenous IGF1R following treatment with IGF-I (lanes labeled +). **C.** Immunoprecipitation with IRS-1 followed by immunoblotting with anti-phosphotyrosine antibody. **D.** Immunoprecipitation with IRS-2 followed by immunoblotting with anti-phosphotyrosine antibody. In panels C and D, lanes 1 and 2 show LCC6-WT and lanes 3 and 4 are LCC6-DN cells. Lanes 5 and 6 are control cells (MCF-7 in panel C and MDA-231BO in panel D), which we have used as positive controls for immunoprecipitations. Lanes 1, 3 and 6 shows untreated cells and lanes 2, 4 and 6 shows cells treated with IGF-I.

FIG. 4. Expression of truncated IGF1R inhibits signaling pathways activated by IGF-I. A and B. Activation of Akt /PKB. Cellular proteins were immunoblotted for phosphorylated Akt (**A**) or total Akt (**B**). **C and D.** Activation of ERK1/ERK2 of the MAPK family. Cells were treated as described for Figure 3 and then immunoblotted for activated ERK1/ERK2 (**C**) or total ERK1/ERK2 (**D**). Both the p42 and p44 kDa ERK1/ERK2 are shown.

In all panels, LCC6-WT cells were untreated (lane 3), or treated with 5 nM IGF-I (lane 4) and LCC6-DN cells were untreated (lane 5) or treated with 5 nM IGF-I (lane 6) for 10 minutes. As controls, we also used MDA-MB 231BO (lanes 1 and 2) and MCF-7 cells (lanes 7 and 8).

FIG. 5. Monolayer growth of LCC6-WT and LCC6-DN cells are similar. LCC6-WT and LCC6-DN cells were grown in monolayers in serum-free conditions were treated with 5 nM IGF-I or 10% fetal bovine serum for 5 days. Cell number was estimated by the MTT assay. Cell number is shown as the absorbance at 570 nm and the data represent the mean ±SEM of four independent experiments with triplicate samples in each experiment.

FIG. 6. Dominant negative IGF1R inhibited IGF-I mediated motility. LCC6-WT, LCC6-DN and MDA-231BO (control) were assayed for motility using a modified Boyden chamber migration assay as described in experimental procedures. Cells were untreated (open bars) or treated with 5 nM IGF-I (filled bars). Results are shown as the total number of migrating cells and the data represent the mean ±SEM of three independent experiments with triplicate samples in each experiment.

FIG. 7. Dominant negative IGF1R inhibited anchorage-independent growth. LCC6-WT and LCC6-DN cells in full FBS containing media were mixed without or with 5nM IGF-I in 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates. Using a grid, colonies formed were counted on a portion of the well. Three randomly selected fields were counted for each well and averaged. Each treatment was done in triplicate and the results are shown as the average number of colonies ±SEM. Experiment was repeated three times with similar results and a representative experiment is shown.

FIG. 8. LCC-WT and LCC6-DN cells formed xenograft tumors in athymic mice.

$5x10^6$ LCC6-WT (n=5) or LCC6-DN cells (n=5) were injected into the mammary fat pad. Tumor growth was measured weekly and is shown as tumor volume calculated using the formula length x $width^2/2$.

FIG. 9. Dominant negative IGF1R inhibited lung metastases.

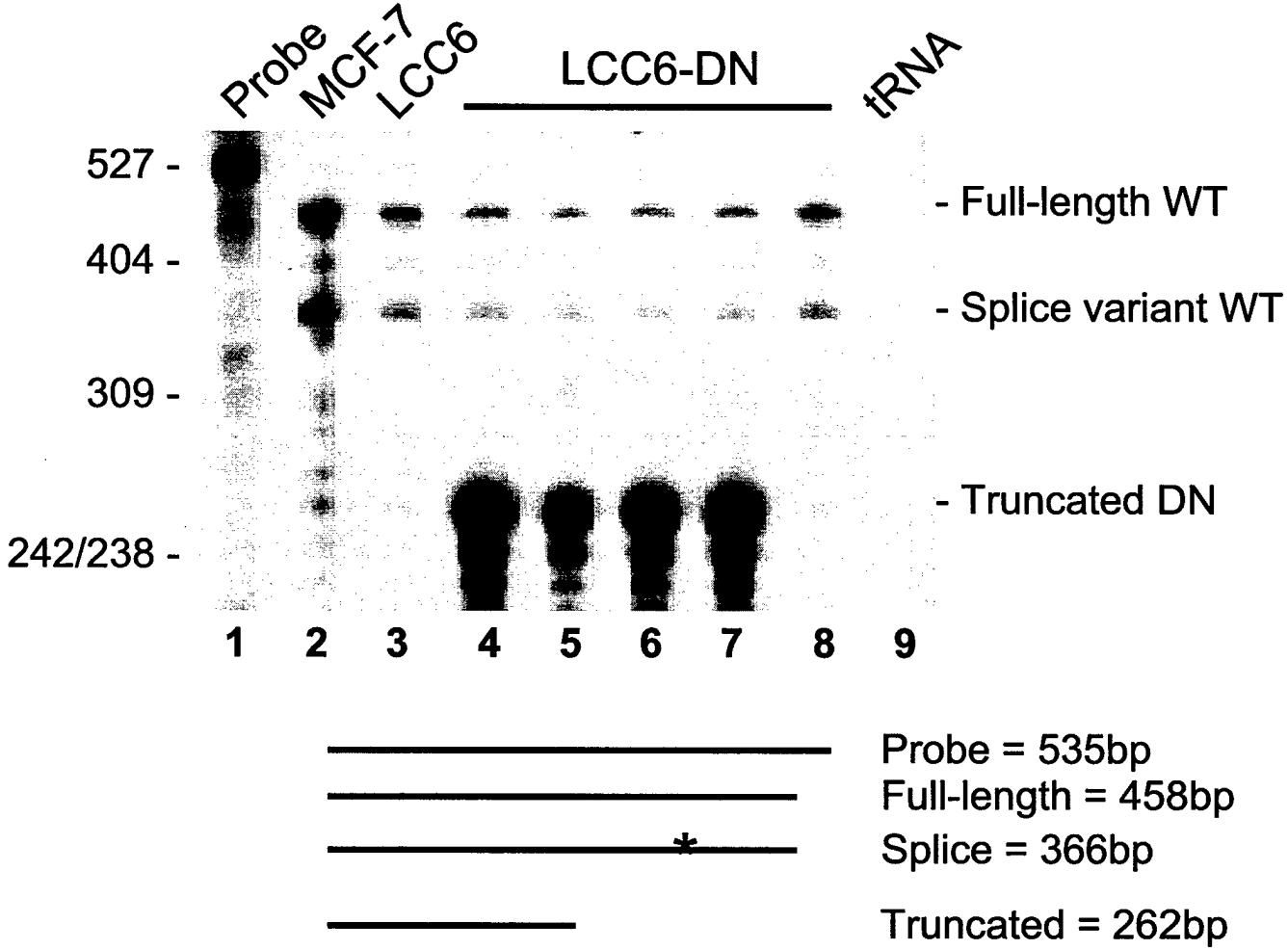
A heart and lung block of a mouse bearing LCC6-WT tumor (left panel) and LCC6-DN tumor (right panel). Mice were injected with LCC6-WT or LCC6-DN cells. The primary tumors were surgically resected at day 21 following injection of cells, and the mice were then sacrificed at day 54 (after injection of cells). Mice were fixed with 1% neutral buffered formalin and the heart and lung were photographed. Multiple nodules measuring ~2 mm in diameter were seen in the lung of LCC6-WT mice. No pulmonary metastases were seen in the lungs of mice injected with LCC6-DN cells.

REFERENCES

1. Kaleko, M., Rutter, W. J., and Miller, A. D. (1990) *Mol Cell Biol* **10**, 464-473
2. Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., DeAngelis, T., Rubin, R., Efstratiadis, A., and Baserga, R. (1994) *Mol Cell Biol* **14**, 3604-3612
3. Sell, C., Rubini, M., Rubin, R., Liu, J. P., Efstratiadis, A., and Baserga, R. (1993) *Proc Natl Acad Sci U S A* **90**, 11217-11221
4. Baserga, R., Sell, C., Porcu, P., and Rubini, M. (1994) *Cell Prolif* **27**, 63-71
5. Wu, Y., Yakar, S., Zhao, L., Hennighausen, L., and LeRoith, D. (2002) *Cancer Res* **62**, 1030-1035.
6. Hadsell, D. L., Greenberg, N. M., Fligger, J. M., Baumrucker, C. R., and Rosen, J. M. (1996) *Endocrinology* **137**, 321-330
7. Hankinson, S. E., Willett, W. C., Colditz, G. A., Hunter, D. J., Michaud, D. S., Deroo, B., Rosner, B., Speizer, F. E., and Pollak, M. (1998) *Lancet* **351**, 1393-1396
8. Pollak, M. (2000) *Eur J Cancer* **36**, 1224-1228
9. Scotlandi, K., Benini, S., Nanni, P., Lollini, P. L., Nicoletti, G., Landuzzi, L., Serra, M., Manara, M. C., Picci, P., and Baldini, N. (1998) *Cancer Res* **58**, 4127-4131
10. Resnik, J. L., Reichart, D. B., Huey, K., Webster, N. J., and Seely, B. L. (1998) *Cancer Res* **58**, 1159-1164
11. Dunn, S. E., Hardman, R. A., Kari, F. W., and Barrett, J. C. (1997) *Cancer Res* **57**, 2687-2693
12. Gooch, J. L., Van Den Berg, C. L., and Yee, D. (1999) *Breast Cancer Res Treat* **56**, 1-10
13. Scotlandi, K., Maini, C., Manara, M. C., Benini, S., Serra, M., Cerisano, V., Strammiello, R., Baldini, N., Lollini, P. L., Nanni, P., Nicoletti, G., and Picci, P. (2002) *Cancer Gene Ther* **9**, 296-307
14. Turner, B. C., Haffty, B. G., Narayanan, L., Yuan, J., Havre, P. A., Gumbs, A. A., Kaplan, L., Burgaud, J. L., Carter, D., Baserga, R., and Glazer, P. M. (1997) *Cancer Res* **57**, 3079-3083
15. Yu, H., and Rohan, T. (2000) *J Natl Cancer Inst* **92**, 1472-1489
16. Sachdev, D., and Yee, D. (2001) *Endocr Relat Cancer* **8**, 197-209
17. Arteaga, C. L., Kitten, L. J., Coronado, E. B., Jacobs, S., Kull, F. C., Jr., Allred, D. C., and Osborne, C. K. (1989) *J Clin Invest* **84**, 1418-1423
18. Li, S. L., Liang, S. J., Guo, N., Wu, A. M., and Fujita-Yamaguchi, Y. (2000) *Cancer Immunol Immunother* **49**, 243-252
19. Doerr, M. E., and Jones, J. I. (1996) *J Biol Chem* **271**, 2443-2447
20. Dunn, S. E., Ehrlich, M., Sharp, N. J., Reiss, K., Solomon, G., Hawkins, R., Baserga, R., and Barrett, J. C. (1998) *Cancer Res* **58**, 3353-3361
21. Andre, F., Rigot, V., Thimonier, J., Montixi, C., Parat, F., Pommier, G., Marvaldi, J., and Luis, J. (1999) *Int J Cancer* **83**, 497-505
22. Long, L., Rubin, R., and Brodt, P. (1998) *Exp Cell Res* **238**, 116-121
23. Stracke, M. L., Engel, J. D., Wilson, L. W., Rechler, M. M., Liotta, L. A., and Schiffmann, E. (1989) *J Biol Chem* **264**, 21544-21549

24. Leventhal, P. S., Shelden, E. A., Kim, B., and Feldman, E. L. (1997) *J Biol Chem* **272**, 5214-5218.
25. Klemke, R. L., Yebra, M., Bayna, E. M., and Cheresh, D. A. (1994) *J Cell Biol* **127**, 859-866
26. Tollefsen, S. E., Stoszek, R. M., and Thompson, K. (1991) *Biochemistry* **30**, 48-54
27. Prager, D., Li, H. L., Asa, S., and Melmed, S. (1994) *Proceedings of the National Academy of Sciences (USA)* **91**, 2181-2185
28. D'Ambrosio, C., Ferber, A., Resnicoff, M., and Baserga, R. (1996) *Cancer Res* **56**, 4013-4020
29. Leonessa, F., Green, D., Licht, T., Wright, A., Wingate-Legette, K., Lippman, J., Gottesman, M. M., and Clarke, R. (1996) *Br J Cancer* **73**, 154-161
30. Cailleau, R., Olive, M., and Cruciger, Q. V. (1978) *In Vitro* **14**, 911-915.
31. Ross, D. T., Scherf, U., Eisen, M. B., Perou, C. M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S. S., Van de Rijn, M., Waltham, M., Pergamenschikov, A., Lee, J. C., Lashkari, D., Shalon, D., Myers, T. G., Weinstein, J. N., Botstein, D., and Brown, P. O. (2000) *Nat Genet* **24**, 227-235
32. Ellison, G., Klinowska, T., Westwood, R. F., Docter, E., French, T., and Fox, J. C. (2002) *Mol Pathol* **55**, 294-299
33. Jackson, J. G., Zhang, X., Yoneda, T., and Yee, D. (2001) *Oncogene* **20**, 7318-7325.
34. Sachdev, D., Li, S. L., Hartell, J. S., Fujita-Yamaguchi, Y., Miller, J. S., and Yee, D. (2003) *Cancer Res* **63**, 627-635
35. Chirgwin, J. M., Przybyla, A. I., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18**, 5294-5299
36. Yee, D., Lebovic, G. S., Marcus, R. R., and Rosen, N. (1989) *The Journal of Biological Chemistry* **264**, 21439-21441
37. Jackson, J. G., White, M. F., and Yee, D. (1998) *J Biol Chem* **273**, 9994-10003
38. Laemmli, U. K. (1970) *Nature* **227**, 680-685
39. Twentyman, P. R., and Luscombe, M. (1987) *Br J Cancer* **56**, 279-285
40. Figueroa, J. A., Sharma, J., Jackson, J. G., McDermott, M. J., Hilsenbeck, S. G., and Yee, D. (1993) *J Cell Physiol* **157**, 229-236
41. Dufourny, B., Alblas, J., van Teeffelen, H. A., van Schaik, F. M., van der Burg, B., Steenbergh, P. H., and Sussenbach, J. S. (1997) *J Biol Chem* **272**, 31163-31171
42. Price, J. E., Polyzos, A., Zhang, R. D., and Daniels, L. M. (1990) *Cancer Res* **50**, 717-721
43. Price, J. E. (1996) *Breast Cancer Research and Treatment* **39**, 93-102
44. Fidler, I. J. (1999) *Cancer Chemother Pharmacol* **43**, S3-10
45. Nicolson, G. L. (1992) *Oncol. Res.* **4**, 389-399
46. Fidler, I. J., Kumar, R., Bielenberg, D. R., and Ellis, L. M. (1999) *Cancer J Sci Am* **4**, S58-S66
47. Chambers, A. F., Groom, A. C., and MacDonald, I. C. (2002) *Nat Rev Cancer* **2**, 563-572
48. Lopez, T., and Hanahan, D. (2002) *Cancer Cell* **1**, 339-353

49. Nishizuka, I., Ishikawa, T., Hamaguchi, Y., Kamiyama, M., Ichikawa, Y., Kadota, K., Miki, R., Tomaru, Y., Mizuno, Y., Tominaga, N., Yano, R., Goto, H., Nitanda, H., Togo, S., Okazaki, Y., Hayashizaki, Y., and Shimada, H. (2002) *Breast Cancer* **9**, 26-32
50. All-Ericsson, C., Girnita, L., Seregard, S., Bartolazzi, A., Jager, M. J., and Larsson, O. (2002) *Invest Ophthalmol Vis Sci* **43**, 1-8
51. Brodt, P., Samani, A., and Navab, R. (2000) *Biochem Pharmacol* **60**, 1101-1107
52. Zhang, X., and Yee, D. (2002) *Cancer Res* **62**, 4369-4375
53. Jones, J. I., Doerr, M. E., and Clemmons, D. R. (1995) *Prog Growth Factor Res* **6**, 319-327
54. Jones, J. I., Prevette, T., Gockerman, A., and Clemmons, D. R. (1996) *Proceedings of the National Academy of Sciences (USA)* **93**, 2482-2487
55. Zheng, B., and Clemmons, D. R. (1998) *Proc Natl Acad Sci U S A* **95**, 11217-11222
56. Van den Berg, C. L., Cox, G. N., Stroh, C. A., Hilsenbeck, S. G., Weng, C. N., McDermott, M. J., Pratt, D., Osborne, C. K., Coronado-Heinsohn, E. B., and Yee, D. (1997) *Eur J Cancer* **33**, 1108-1113
57. Reinmuth, N., Fan, F., Liu, W., Parikh, A. A., Stoeltzing, O., Jung, Y. D., Bucana, C. D., Radinsky, R., Gallick, G. E., and Ellis, L. M. (2002) *Lab Invest* **82**, 1377-1389
58. Mauro, L., Bartucci, M., Morelli, C., Ando, S., and Surmacz, E. (2001) *J Biol Chem* **22**, 22
59. Mauro, L., Salerno, M., Morelli, C., Boterberg, T., Bracke, M. E., and Surmacz, E. (2003) *J Cell Physiol* **194**, 108-116
60. Satyamoorthy, K., Li, G., Vaidya, B., Kalabis, J., and Herlyn, M. (2002) *Cell Growth Differ* **13**, 87-93
61. Hailey, J., Maxwell, E., Koukouras, K., Bishop, W. R., Pachter, J. A., and Wang, Y. (2002) *Mol Cancer Ther* **1**, 1349-1353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tRNA
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404 -
309 -
242/238 -
- Full-length WT
- Splice variant WT
- Truncated DN
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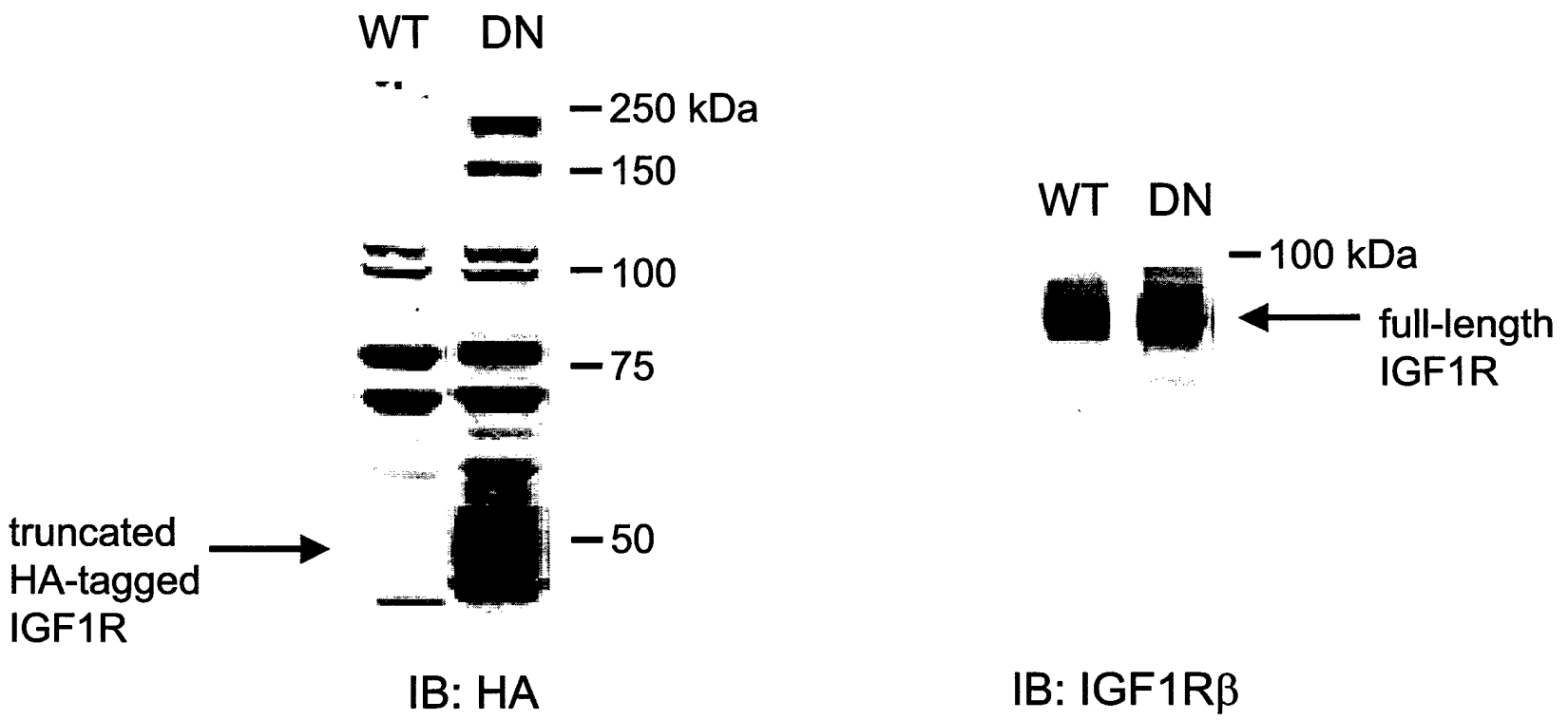
Probe = 535bp
Full-length = 458bp
Splice = 366bp
Truncated = 262bp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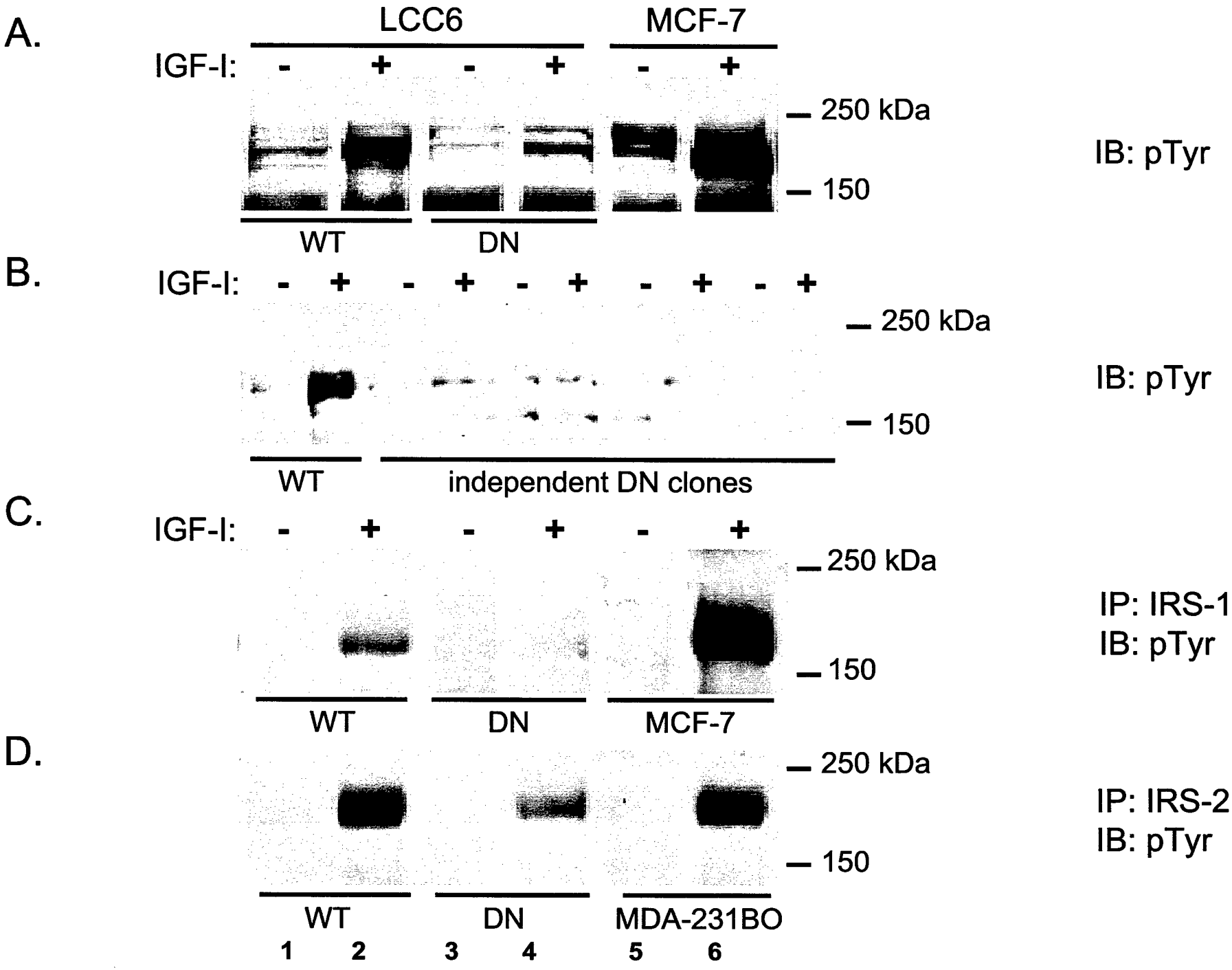
A.
LCC6
MCF-7
IGF-I: - + - + - +
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150
IB: pTyr
WT
DN
B.
IGF-I: - + - + - + - + - +
250 kDa
IB: pTyr
150
WT
independent DN clones
C.
IGF-I: - + - + - +
250 kDa
IP: IRS-1
IB: pTyr
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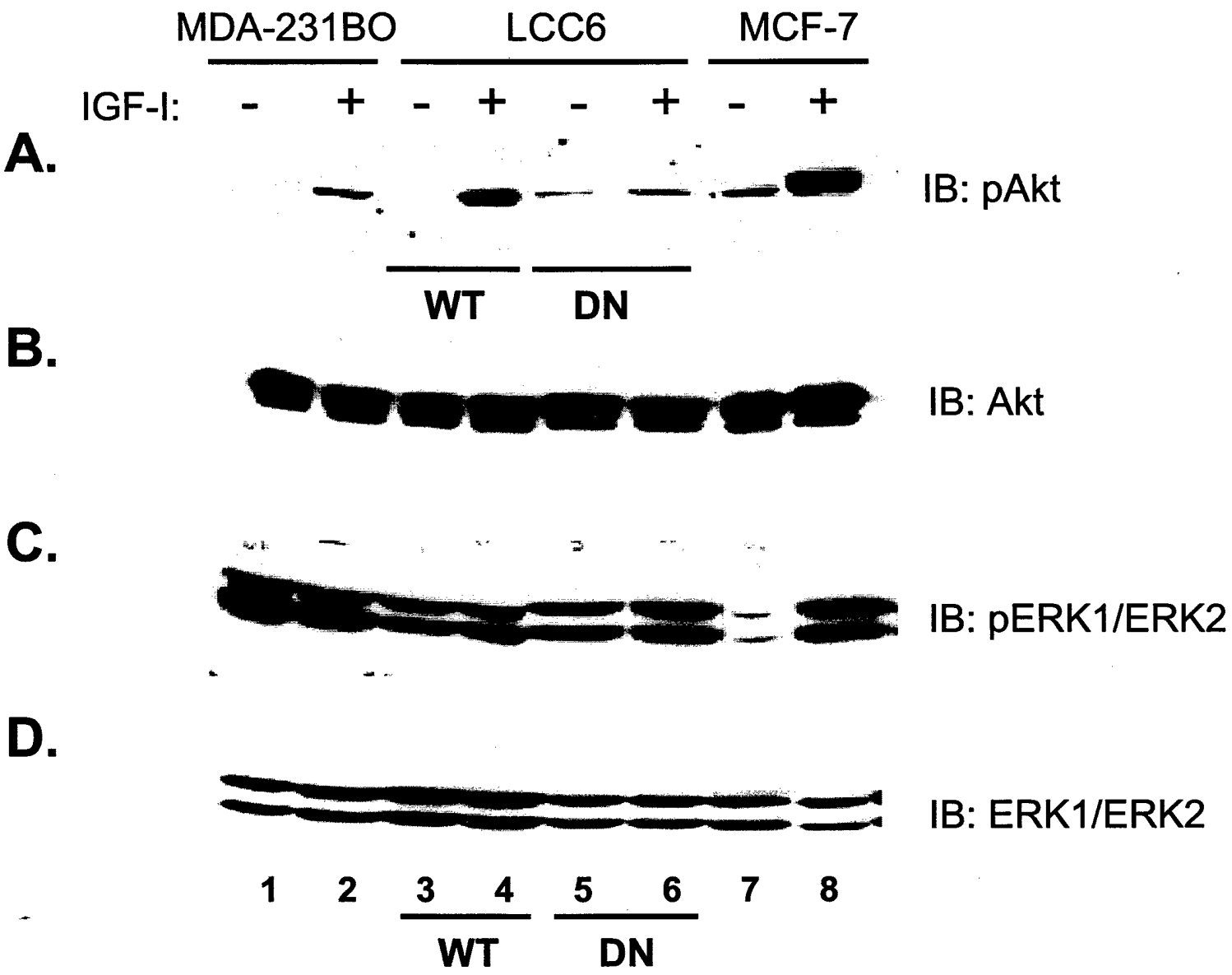
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IB: pAkt
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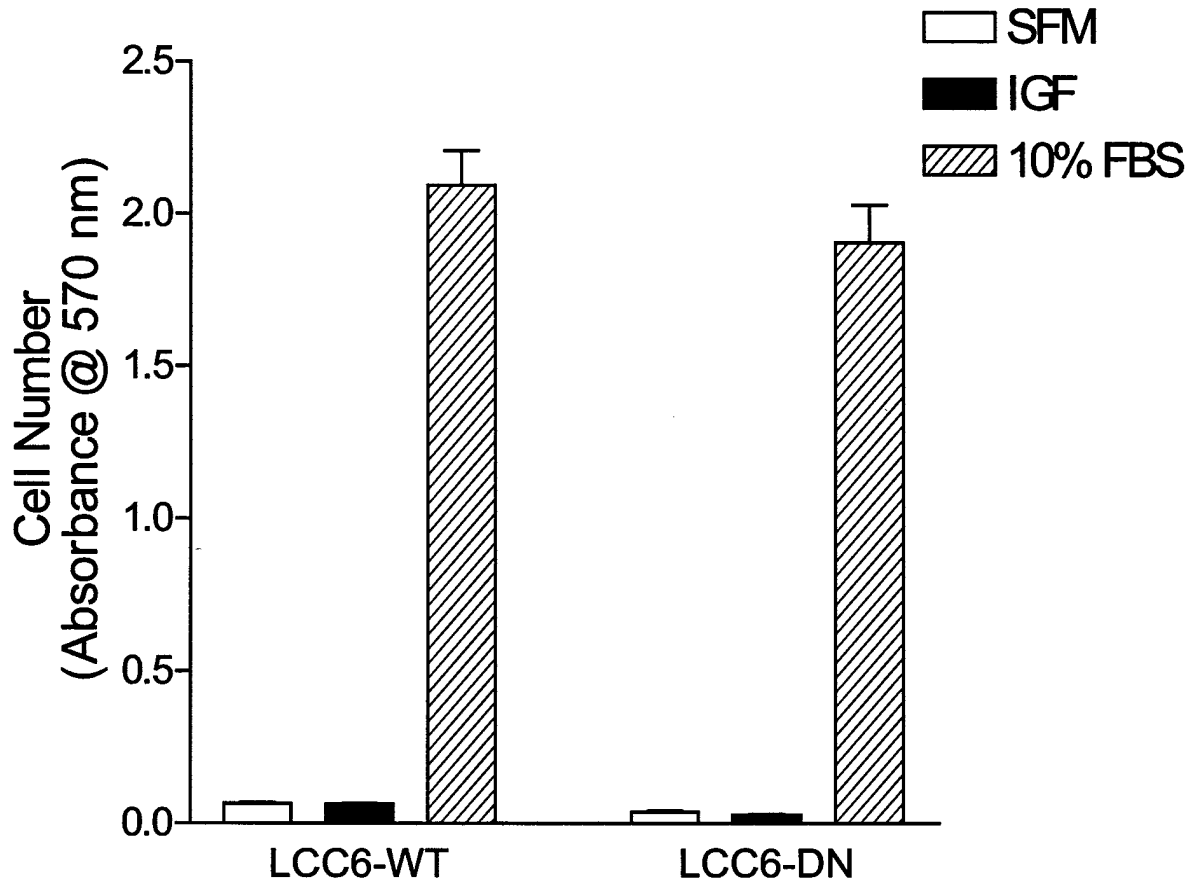
WT
DN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IGF
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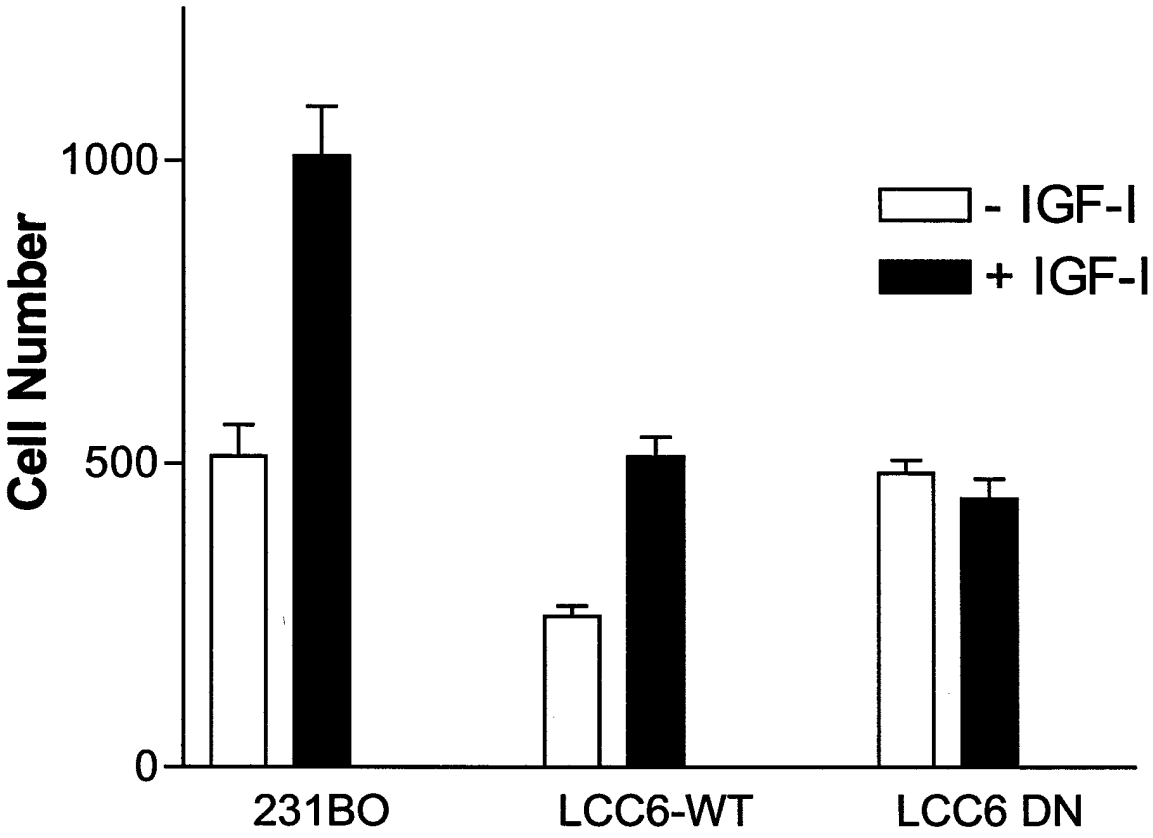
LCC6-WT
LCC6-DN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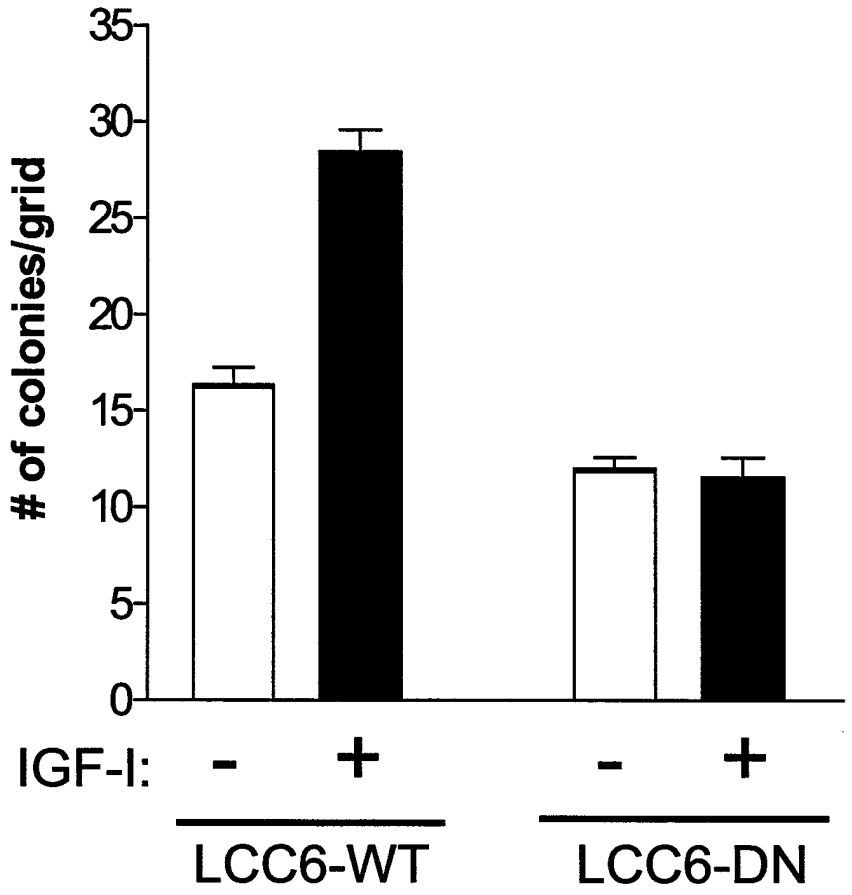
LCC6 DN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5
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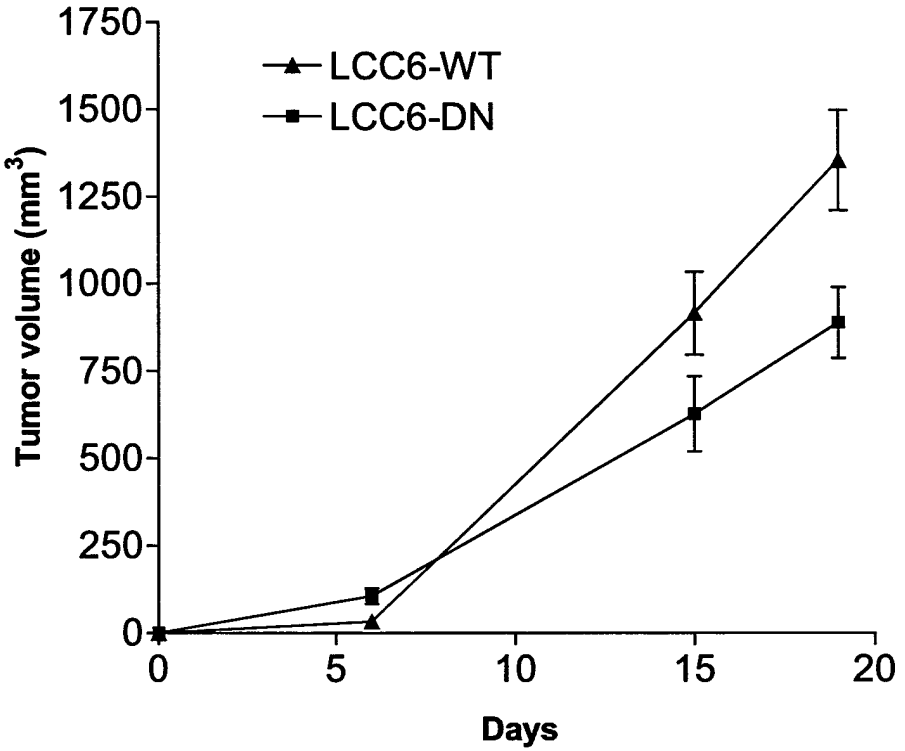
IGF-I:
-
+
-
+
LCC6-WT
LCC6-DN


LCC6-WT
LCC6-DN
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1000
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500
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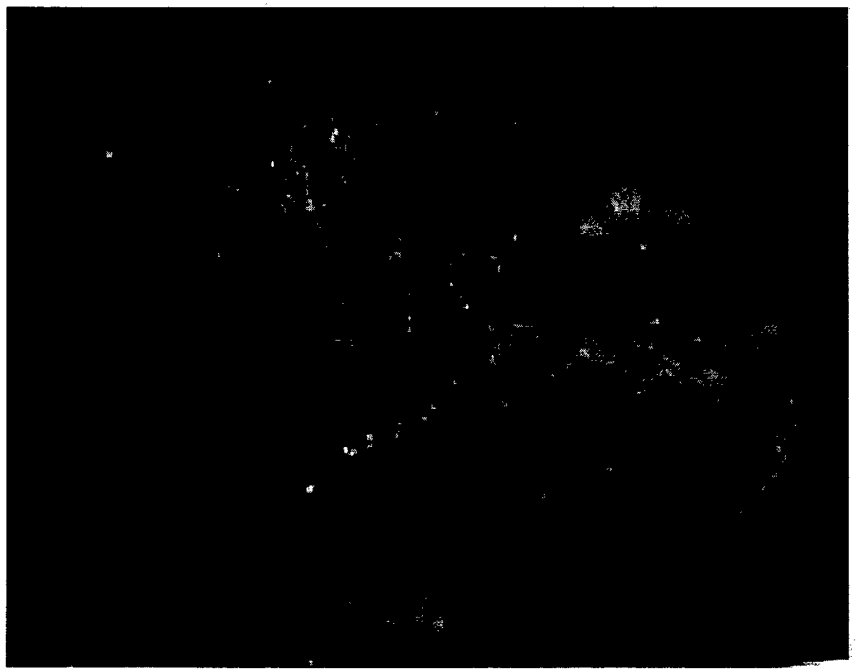
0
0
5
10
15
20
Days

Day 54



LCC6-WT

LCC6-DN